Different Requirements for Signal Transducer and Activator of Transcription 1α and Interferon Regulatory Factor 1 in the Regulation of Low Molecular Mass Polypeptide 2 and Transporter Associated with Antigen Processing 1 Gene Expression*

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The components of the antigen processing machinery, low molecular mass polypeptide (LMP) 2 and transporter associated with antigen processing (TAP) 1, are encoded by closely linked genes within the major histocompatibility complex class II subregion. Although the two genes share a bi-directional promoter, LMP2 and TAP1 have differential cellular expression. TAP1 is expressed constitutively. However, LMP2 expression requires induction by interferon-γ in most cells. The regulatory elements within the LMP2/TAP1 promoter and the transcription factors that bind these elements have been defined. However, how these transactivators regulate differential TAP1 and LMP2 gene transcription is not known. We have addressed this question by analyzing three human melanoma cell lines with distinct phenotypes of LMP2 and TAP1 expression. Whereas binding of either interferon regulatory factor 1 or Stat1 to the overlapping interferon consensus sequence-2/GAS is sufficient for regulating transcription of the TAP1 gene, binding of both factors is required for LMP2 gene transcription. This conclusion is supported by restoration of LMP2 gene transcription following transfection of wild type Stat1α or interferon regulatory factor 1 cDNA into cells lacking these transcription factors. The flexibility in the regulation of the TAP1 gene may reflect its role in maintaining immune surveillance. Furthermore, lack of LMP2 gene transcription in quiescent human cells suggests that LMP2 expression reflects a state of cell activation.

The transporter associated with antigen processing (TAP)1 and TAP2 gene products are responsible for ATP-dependent transport of peptides from the cytosol to the endoplasmic reticulum where peptides bind to newly synthesized MHC class I molecules (1). These peptides are generated mostly from endogenous proteins by the activity of a multi-subunit, cytosolic proteasome complex (2). Incorporation of the low molecular mass polypeptide (LMP) 2 and LMP7 subunits into the proteasome complex increases the amount of generated peptides available for binding to MHC class I antigens (3–6). Expression of the TAP and LMP genes can be up-regulated by immune regulators such as interferon (IFN)-γ leading to an increase in processing and presentation of MHC class I restricted antigens (7–9).

The involvement of LMP and TAP proteins in MHC class I-dependent antigen processing and the close spatial relationship of the corresponding genes within the MHC class II region have stimulated interest in the characterization of the mechanisms regulating their expression. Transition of LMP2 and TAP1 genes is regulated by a 593-bp promoter/enhancer region located between the LMP2 and TAP1 genes that is thought to be both necessary and sufficient for regulating coordinate transcription of the two genes (10). This promoter contains five cis-regulatory sequences. They include SP-1, αB, interferon consensus sequence (ICS)-1, ICS-2, and γ-activated sequence (GAS). DNA binding activity to the GC-rich SP-1 sequence is required for constitutive expression of both LMP2 and TAP1 genes, whereas that to the αB site only slightly affects constitutive transcription of the two genes. The ICS-1 region is also important for constitutive expression of the TAP1 gene. In contrast, the ICS-2 and GAS sites have not been implicated in constitutive transcription of the TAP1 gene (11). To the best of our knowledge, the roles of the ICS-2 and GAS sequences in regulating constitutive transcription of the LMP2 gene have not been analyzed.

Enhancement of LMP2 and TAP1 transcription in HeLa cells by tumor necrosis factor-α and IFN-γ is mediated by different transcription factors. Up-regulation of TAP1 gene expression by tumor necrosis factor-α requires factor binding only to the αB sequence (11), whereas increased LMP2 gene transcription requires factor binding to both SP-1 and αB sequences (10). Rapid induction of the TAP1 gene in HeLa cells incubated with IFN-γ (11) requires binding of the IFN-γ-induced transcription factor, Stat1α, to GAS. Furthermore, IFN-γ-mediated LMP2 and TAP1 gene expression is also induced by binding of interferon regulatory factor (IRF) 1 to the ICS-2 region (12). The role of GAS in IFN-γ-induced transcription of the LMP2 gene remains to be determined.

Previous studies have demonstrated coordinate constitutive expression of LMP2 and TAP1 genes in HeLa cells (10), in
human small cell lung carcinoma cell lines (13), and in human renal carcinoma cell lines (14). We have observed this phenotype in about 50% of a panel of cultured human melanoma cell lines. However, most of the remaining melanoma cell lines analyzed, as well as cultured melanocytes and fibroblasts, constitutively express only TAP1 protein. Transcription of the LMP2 gene in fibroblasts, in melanocytes, and in about 50% of melanoma cell lines that do not constitutively express LMP2 was induced following incubation with IFN-γ. This observation implies the role of IFN-γ-inducible factors in transcriptional regulation of the LMP2 gene. To the best of our knowledge, these factors have not been identified yet. Therefore, in the present study we have characterized the IFN-γ-inducible trans-acting factors required for LMP2 gene transcription, because this information contributes to our understanding of the molecular mechanism(s) regulating the antigen presentation machinery. To this end, we have analyzed the melanoma cell lines 526, 624.c, and 677, which constitutively express TAP1 but exhibit three distinct phenotypes of LMP2 gene expression. LMP2 is constitutively expressed only in the 677 cell line, is inducible in 624.c cells following incubation with IFN-γ, and is not detectable in 526 cells even following incubation with IFN-γ. The present study demonstrates for the first time that discordant LMP2 and TAP1 gene transcription reflects differential requirements of Stat1α and IRF1.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The melanoma cell lines, 526, 624.c, and 677, which constitutively express TAP1 but exhibit three distinct phenotypes of LMP2 gene expression, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Plasmids**—The 593-bp inserts from the p33luc and p34luc plasmids (14) containing the minimal LMP2/TAP1 promoter oriented in the direction of TAP1 and LMP2, respectively, were obtained following digestion with KpnI and XhoI. The p33SEAP and p34SEAP reporter constructs were obtained by subcloning the p33 and p34 KpnI/XhoI inserts into the pSEAP2-enhancer plasmid (CLONTECH Inc., Palo Alto, CA). The BamHI/EcoRV insert containing the Stat1(p91) cDNA was excised from pStat1 (19) and subcloned into the mammalian expression vector pcDNA3.1 (CLONTECH Inc.) to obtain pcStat1. Construction of the murine IRF1 (p-act1) and IRF2 (p-act2) expression vectors has been described elsewhere (18).

**Role of Stat1α and IRF1 in LMP2 and TAP1 Gene Transcription**

**Synthetic oligonucleotides used for PCR amplification of LMP and TAP cDNA and for analysis of the LMP/TAP promoter in EMSA**

| Oligonucleotide | Sequence (5′→3′) |
|-----------------|-----------------|
| Primers for PCR | TCCCCTGGGATTC | CACCTCGAGGCTTTCGA |
| LMP7 reverse    | CCGTACCATGGCAGGAGAGC | CGGTCCTGAGTTCGAGAGC |
| TAP1 reverse    | CGGTCCTGAGTTCGAGAGC | CGGTCCTGAGTTCGAGAGC |
| TAP2 forward    | CGGTCCTGAGTTCGAGAGC | CGGTCCTGAGTTCGAGAGC |
| TAP2 reverse    | CAAATGTTAGATCAGTTC | CGGTCCTGAGTTCGAGAGC |
| Probes for EMSA | TGGCGCTCTGCCCACTTCAATAGG | TGGCGCTCTGCCCACTTCAATAGG |
| GAS (LMP/TAP)   | GCGGCCGCGTCTTTCGA | GCGGCCGCGTCTTTCGA |
| ICS-1 (LMP/TAP) | GGGAAAGCGAAATCGATTC | GGGAAAGCGAAATCGATTC |
| ICS-2 (LMP/TAP) | GCGGCCGCGTCTTTCGA | GCGGCCGCGTCTTTCGA |
| κB (LMP/TAP)    | GGGAAAGCGAAATCGATTC | GGGAAAGCGAAATCGATTC |
| SP-1 (LMP/TAP)  | GGGAAAGCGAAATCGATTC | GGGAAAGCGAAATCGATTC |

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were performed using nuclear extracts isolated from untreated and IFN-γ-treated cells and oligonucleotides corresponding to ICS-1, IFN-γ-induced factor (ICSI-1), IFN-γ-induced factor (ICS-2), and Sp1. Equal amounts of nuclear extract were incubated with 32P-labeled oligonucleotide. After electrophoresis, autoradiography was performed using X-ray film. EMSA supershift assays were performed like EMSA except that the cold oligonucleotide was added to the reaction mixture simultaneously at a concentration 50-fold that of the corresponding radiolabeled oligonucleotide. Supershift assays were performed like EMSA except that the nuclear extracts were incubated on ice for 20 min with antibodies specific for the indicated transcription factor (1 μg) before incubation with nuclear extract.
incubation with IFN-\(\gamma\). Expression of the reporter gene in cells transfected with p33SEAP indicated that the exogenously provided promoter was active in the direction of TAP1 transcription in 526, 624.c, and 677 cells. Expression of the reporter gene following transfection of p34SEAP was consistently higher than that following transfection of p34SEAP. These findings are compatible with aberrations in expression and/or function of trans-acting factor(s) as a mechanism for the lack of LMP2 gene transcription in 526 and 624.c cells. These results also show that activation of IFN-\(\gamma\)-inducible factor(s) restore promoter activity in the direction of LMP2 transcription in 624.c cells.

**Lack of Stat1\(\alpha\) or IRF1 Binding at the LMP2/TAP1 Promoter Is Associated with Lack of LMP2 Gene Transcription in 526 and 624.c Human Melanoma Cell Lines**—We then examined by EMSA the binding activity of transcription factors to the GAS, ICS-2, ICS-1, \(\kappa\)B, and SP-1 cis-regulatory regions of the TAP1/ LMP2 promoter (Fig. 3) in untreated and IFN-\(\gamma\)-treated 526, 624.c, and 677 melanoma cell lines. A single binding activity was observed in the three cell lines when an oligonucleotide corresponding to the upstream ICS-1 was used as a probe. Binding activity to the \(\kappa\)B sequence in the TAP1/LMP2 promoter region was observed in the three cell lines. Gel mobility shift assays with the SP-1 oligonucleotide showed two DNA bound complexes in the three cell lines (Fig. 4A). Binding activities to the ICS-1, \(\kappa\)B, and SP-1 regions were not affected by incubation of cells with IFN-\(\gamma\).

Differential GAS binding activities were observed in 526, 624.c, and 677 cell lines (Fig. 4B). Protein-DNA complexes corresponding to GAS binding activity observed in 624.c and 677 cells were not detected in 526 cells even after incubation with IFN-\(\gamma\). The binding activity to GAS observed in both 624.c and 677 cells was competed away by using a \(50 \times\) concentration of a consensus Stat1\(\alpha\) binding oligonucleotide as a cold competitor in EMSA. Furthermore, super shift assays showed that this band was competed away by anti-Stat1\(\alpha\) antibodies in both 624.c and 677 cells. Therefore, the lack of LMP2 mRNA in 526 cells is associated with the absence of Stat1\(\alpha\) binding to GAS.

Nuclear extracts from the 526, 624.c, and 677 cell lines displayed one major binding activity to the ICS-2 probe. Anti-IRF1 antibodies super shifted the protein-DNA complex in 526 and 677 cells, whereas anti-IRF2 antibodies super shifted the protein-DNA complex in 624.c cells (Fig. 4B). Thus, most of the binding activity to ICS-2 corresponds to IRF1 in 526 and 677 cells and to IRF2 in 624.c cells. Absence of IRF1 binding to ICS-2, which is replaced by IRF2 binding activity in 624.c cells, is associated with lack of LMP2 gene transcription in these cells.

**LMP2 Gene Transcription Depends on Stat1\(\alpha\) Expression and Function in 526 and U3A Human Cell Lines**—Stat1\(\alpha\) could not be detected by Western blot analysis in 526 cells but was constitutively expressed in 677 cells (Fig. 5A). Further, Stat1\(\alpha\) expression could not be detected in 526 cells following incubation with IFN-\(\gamma\) (data not shown). Therefore, to determine whether lack of LMP2 gene transcription observed in 526 cells is a distinct phenotype of Stat1\(\alpha\)-deficient cells, LMP2 gene transcription was analyzed in U3A cells. The latter cell line is a Stat1\(\alpha\)-deficient mutant derived from the human fibrosarcoma cell line 2fhGH that constitutively expresses Stat1\(\alpha\) (15) (Fig. 5A). S1 nuclease-protected bands corresponding to LMP2 mRNA were not detected in the Stat1\(\alpha\)-deficient 526 and U3A cells. Both 677 and 2fhGH cells transcribe the LMP2 gene. TAP1 mRNA was detected in all four cell lines (Fig. 5B). These results show that Stat1\(\alpha\)-deficient U3A and 526 cells do not transcribe the LMP2 gene.
To conclusively prove that absence of Stat1α causes lack of LMP2 gene transcription in the Stat1α-deficient 526 and U3A cells, the two cell lines were transiently transfected with cDNA encoding Stat1α (Fig. 5C). S1 nuclease-protected bands corresponding to LMP2 were detected in the two transfected cell lines. TAP1 gene transcription did not change markedly in 526 and U3A cells following transfection with Stat1α (p91) cDNA (Fig. 5D). Over-expression of IRF1 Restores LMP2 Gene Transcription in 624.c Human Melanoma Cell Line—Appearance of IRF1 binding activity following incubation of 624.c cells with IFN-γ was associated with LMP2 gene transcription (data not shown). To ascertain whether the absence of IRF1 binding activity is primarily responsible for the lack of constitutive LMP2 gene transcription in 624.c cells, cDNA encoding wild type IRF1 was transiently transfected into these cells (Fig. 5A). Expression of IRF1 and IRF2 in mock transfected and in IRF1- and IRF2-transfected cells is shown in Fig. 6B. S1 nuclease-protected bands corresponding to LMP2 mRNA were detected in 624.c cells transfected with IRF1 (Fig. 6A). The level of LMP2 message in IRF1-transfected 624.c cells was similar to that induced following incubation of untransfected 624.c cells with IFN-γ. Changes in the level of TAP1 mRNA were not significant following transfection of cDNA encoding wild type IRF1.

As shown in Fig. 4B, IRF2 binds at the ICS-2 site in 624.c cells. To demonstrate that IRF2 competing for IRF1 binding at the ICS-2 site abrogates LMP2 gene transcription, 624.c cells were transiently transfected with cDNA encoding IRF2 and then incubated with IFN-γ. The level of LMP2 mRNA in these 624.c cells was reduced by more than 50% compared with that detected in untransfected, IFN-γ-treated 624.c cells. The level of TAP1 mRNA was decreased by ~5% following transfection.
Stat1α monoclonal antibody followed by horseradish peroxidase-conjugated goat anti-mouse IgG antibodies. Blots were developed using the ECL reagent (Amersham Pharmacia Biotech). Poly(A)^+ RNA was isolated from mock transfected (with pcDNA3) (B) or Stat1α-transfected (with p-act1) (D) 526, 677, U3A, and 2fGH cells. RNA samples were then analyzed in S1 nuclease protection assays using single-stranded probes for LMP2 (641 bp), LMP7 (796 bp), TAP1 (958 bp), TAP2 (1056 bp), and β-actin (838 bp). The probes for β-actin and TAP were used in separate reactions because they have similar mobility. A fine band of mobility slightly higher than that of the β-actin band was detected in some samples depending on the RNA preparation.

with cDNA encoding wild type IRF2 (Fig. 6A). Thus, lack of LMP2 gene transcription is primarily because of the replacement of IRF1 by IRF2 binding at ICS-2 in 624.c cells.

**DISCUSSION**

Analysis of the 526, 624.c, and 677 melanoma cell lines has shown distinct phenotypes of LMP2 and TAP1 gene transcription. The TAP1 gene is constitutively transcribed in the three cell lines, whereas the LMP2 gene is constitutively transcribed only in 677 cells. LMP2 gene transcription is induced by IFN-γ in 624.c cells but not in 526 cells. Differential transcription of LMP2 in the three cell lines is associated with different binding activities at the common promoter that regulates transcription of both LMP2 and TAP1 genes. Nuclear extracts from 677 cells that transcribe both LMP2 and TAP1 genes exhibit binding activity to the ICS-1, ICS-2, GAS, αβ, and SP-1 sites of the LMP2/TAP1 promoter. In contrast, nuclear extracts from 526 cells, that do not transcribe the LMP2 gene even after incubation with IFN-γ selectively lack binding activity of Stat1α to GAS. The lack of binding activity to GAS in 526 cells is because of absence of Stat1α gene expression. The ICS-2 binding activity corresponds to IRF1 in both 526 and 677 cells but corresponds to IRF2 in 624.c cells that transcribe the LMP2 gene only following incubation with IFN-γ. These results demonstrate that binding of both Stat1α and IRF1 to the overlapping ICS-2/GAS site within the LMP2/TAP1 promoter is required for LMP2 gene transcription. Furthermore, simultaneous binding of Stat1α and IRF1 is required for coordinate transcription of the LMP2 and TAP1 genes. These conclusions are supported by two additional lines of evidence. Transfection of cDNA encoding Stat1α (p91) into Stat1α-deficient U3A and 526 cells restores LMP2 gene transcription in these cell lines. Furthermore, transfection of IRF1 induces LMP2 gene transcription in 624.c cells. TAP1 gene transcription is not affected in these experiments. Basal transcription of TAP1 is independent of binding activity at either GAS or ICS-2 (11). Because there is no significant increase in levels of TAP1 mRNA following IRF1 over-expression in 624.c cells, it appears that either Stat1α or IRF1 is sufficient for maximal transcription of the TAP1 gene. Our interpretation suggests that the coordinate expression of LMP2 and TAP1 described in HeLa cells, and in certain human lung and renal carcinoma cell lines (10, 13, 14) and observed by us in the 677 melanoma cell line, reflects activation of cells in response to extracellular and/or intracellular stimuli. This result is in binding of both Stat1α and IRF1 to the promoter of the LMP2 and TAP1 genes. The differential expression of LMP2 and TAP1 in 624.c cells, which is similar to the phenotype observed in cultured human melanocytes and fibroblasts, is compatible with a quiescent state of cells. This model is also in...
agreement with the hypothesis that LMP2 expression is not essential for survival of normal cells (20).

Differential transcription of LMP2 and TAP1 genes from a common promoter parallels similar findings in other closely linked genes that are divergently transcribed from a single promoter. The H2A and H2B genes in the Xenopus Xh3 histone gene cluster are divergently transcribed because of the activity of overlapping promoter sequences that share multiple regulatory elements (21). Furthermore, the human and murine COL4A1 and COL4A2 genes are expressed discordantly, although they share the same promoter. Lack of binding activity at the CCAAT and CTC regions markedly reduces transcription of the COL4A2 gene without affecting that of the COLA1 gene (22). The present study suggests that the intragenic promoter between the LMP2 and TAP1 genes is indeed sufficient to induce transcription in both directions, provided that the TAP1 proximal SP-1 and eB sites are occupied. Whether both LMP2 and TAP1 genes are expressed or only the TAP1 gene is transcribed appears to depend on transcription factor binding at IC-2/GAS.

Binding of Stat1α in 624.c and 677 melanoma cell lines in the basal state is noteworthy in view of the previously reported requirement for Stat1α binding to GAS for immediate response to IFN-γ in HeLa cells (11). The phenotypes of Stat1α expression and activation described in this study are, however, not unique to the cell lines we have characterized. Activated Stat-related factors have been observed in normal cells, in cells isolated from patients with malignancies, as well as in several tumor cell lines (23–25). The differential constitutive expression of LMP2 and TAP1 we have found in the Stat1α-deficient 526 melanoma cells and U3A fibrosarcoma cells has also been observed in the melanoma cell line SK-MEL-3 (26) and in quiescent human fibroblasts, which do not have constitutively active Stat1α (27). Binding activity of Stat1α and LMP2 gene transcription can be induced in SK-MEL-3 cells (26, 27) and human fibroblasts following incubation with IFN-γ. Whether this regulation is conserved phylogenetically is not known, because to the best of our knowledge, LMP2 gene expression has not been investigated in Stat1α−/− mice.

As observed earlier in HeLa cells (11, 12), the ICS-2, which contains a near consensus IRF binding site (IRF-E), is bound by IRF1 in 526 and 677 cells. In contrast, this sequence is bound by IRF2 in 624.c cells. IRF1 and IRF2 can compete for binding to the IRF-E sequences found in IFN-inducible promoters because both proteins have almost identical DNA-binding domains (28, 29). The resulting phenotypes, however, are markedly different, because IRF1 is a transcriptional activator, whereas IRF2 exerts negative regulatory functions via its transactivation domain (30). That IRF2 acts as a negative regulator of LMP2 gene transcription in untreated 624.c cells has been validated by the detection of LMP2 mRNA in these cells following transfection with cDNA encoding IRF1. Furthermore, reduction of LMP2 and TAP1 mRNA levels by 85 and ~5%, respectively, in IFN-γ-treated 624.c cells transfected with IRF-2 resembles the marked reduction of TAP1 and especially that of LMP2 mRNA in tissues of IRF1−/− mice (12). The low level of LMP2 mRNA detected in IRF1−/− mice may reflect species differences in regulation of the mouse and human LMP2/TAP1 genes. Alternatively, it may reflect the activity in vivo of other IRF-like factors that bind to the IRF-E site and partially substitute for IRF1 (12).

Discordant expression of LMP2 and TAP1 may reflect the different roles of the two proteins in maintenance of immune surveillance and during an immune response. The level of LMP2 expression appears to affect the magnitude of the immune response, because absence of the LMP2 subunit in the proteasome decreases the efficiency of presentation of certain viral epitopes (31). In addition, IFN-γ-induced incorporation of LMP2 into the proteasome increases the amount of antigenic peptides available for binding to MHC class I molecules (32). The absence of a functional TAP heterodimer has more far-reaching effects on immune recognition. TAP1−/− mice have low cell surface MHC class I antigen expression and a reduced number of CD8+ T cells, markedly attenuating their ability to mount an anti-viral response (33). Lack of a functional TAP heterodimer in humans also results in very low MHC class I antigen expression at the cell surface. Patients with this abnormality present with all the symptoms of bare lymphocyte syndrome (34). Although TAP-independent transport of peptide epitopes released by signal peptide activity is known, a majority of known peptides require a functional TAP heterodimer for transport into the endoplasmic reticulum (35). The different functional characteristics of LMP2 and TAP1 may thus account for the selective constitutive expression of TAP1 in quiescent cells.

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