HLA-A2 and HLA-B7 antigens are phosphorylated in vitro by Rous sarcoma virus kinase (pp60V-src) at a tyrosine residue encoded in a highly conserved exon of the intracellular domain

(COOH-terminal domain homology/tyrosine conservation/retrovirus)

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ABSTRACT HLA-A2 and -B7 antigens are phosphorylated by Rous sarcoma kinase (pp60V-src) in vitro. The phosphate group is attached to the heavy chains as determined by NaDodSO4/polyacrylamide gel electrophoresis. The site of phosphorylation was localized to the COOH-terminal intracellular domain by its susceptibility to limited trypsin proteinolysis. Furthermore, the 32P-labeled amino acid is a single tyrosine residue located in the COOH terminus of the heavy chain. The protein sequences of known class I human and murine intracellular domains contain a highly conserved sequence -K-G-G-X-Y- located NH2-terminally to the single tyrosine residue of this domain. The DNA sequences that encode class I antigen intracellular domains were compared by computer with a homology matrix program. Exon 6 which encodes the conserved tyrosine-containing protein sequence in both human and mouse is 75% homologous across species and 90-100% homologous within species. The significance of the high degree of conservation within exon 6 is discussed.

The HLA-A and -B antigens are the best structurally characterized transmembrane proteins of nucleated cells. These class I molecules are encoded within the major histocompatibility complex and are intimately involved in cellular immune regulation. They are present as cell surface glycoproteins, composed of a polymorphic 44,000-dalton heavy chain and an invariant noncovalently associated subunit termed β2-microglobulin (β2M). The bulk of the heavy chain and β2M are located on the extracellular portion of the cell membrane where the antigenic structure of the molecule is presented. The extracellular domain of the heavy chain is linked by its hydrophobic transmembrane segment to a hydrophilic 30-amino acid residue COOH-terminal intracellular domain. Assessment of the role of this intracellular domain in class I antigen function has been the subject of a recent review (1). Phosphorylation of serine(s) within the intracellular domain has been demonstrated for HLA-A and -B antigens both in vivo and in vitro in normal and Epstein–Barr virus-transformed lymphocytes (2). H-2, the equivalent class I antigen of the murine system, is also known to be phosphorylated in vivo at serine(s) of the intracellular domain (3). Phosphorylation has been shown to play a role in the regulation of interactions among cytoskeletal proteins (4). Biochemical evaluation of interactions between class I antigens and the cytoskeleton (5, 6) may help to unravel the mechanisms regulating HLA distribution at the cell surface.

The Rous sarcoma virus contains a gene, designated src, which encodes a 60,000-dalton phosphoprotein (pp60V-src) responsible for the neoplastic transformation of cells infected with the virus (7). The protein kinase activity associated with pp60V-src has been shown to phosphorylate tyrosine residues in various substrates (8, 9). Uninfected vertebrate cells contain an endogenous pp60V-src homologue designated pp60V-src which possesses similar antigenic and functional properties (10, 11). The available data on pp60V-src indicate that this kinase is associated with the cytoplasmic face of the cell surface membrane (12), where the intracellular domains of class I antigens are also located. The protein sequences for the COOH-terminal intracellular domains of HLA-A2 and -B7 have been determined, and they each contain a single tyrosine residue at amino acid position 390 (13).

To determine whether class I antigens might be substrates for a tyrosine kinase we have conducted an in vitro phosphorylation study using pp60V-src and purified, detergent-soluble HLA-A2 and -B7 antigens. Both HLA-A2 and -B7 were labeled at a single tyrosine residue of the intracellular domain. Examination of the DNA sequences encoding the intracellular domains of human and murine class I antigens showed that one of the three exons encoding the 30-amino acid intracellular domain is highly conserved both within and across species. It is this exon which encodes a 10-amino acid stretch containing the tyrosine residue phosphorylated by pp60V-src.

MATERIALS AND METHODS

Materials. JY transformed human lymphoblastoid cells (homozgyous HLA-A2 and -B7) were used as the source of HLA antigens. Allospecific mouse monoclonal antibodies PA2.1 and BB7.1 were provided by Peter Parham. pp60V-src was prepared by immunopurification on chromatography as described (14, 15). Tubulin was prepared from rabbit brain by the method of Blobdr et al. (16). Carrier-free sodium (32P)orthophosphate was obtained from New England Nuclear. [γ-32P]ATP was prepared by the method of Johnson and Walseth (17). Trypsin (diphenylcarbamoyl chloride-treated), phenylmethylsulfonyl fluoride, and α-casein were from Sigma. Sepharose CL-4B was from Pharmacia. Detergents Brij (a 2:1 mixture of Brij 99 and 97) from Emulsion Engineering (Elk Grove, IL), Nonidet P-40 from Particle Data Laboratories (Elmhurst, IL), and deoxycholate from Schwarz/Mann were used in the purification of HLA antigens. Phosphoserine and phosphothreonine were obtained from Sigma; phosphotyrosine was prepared by the method of Plimmer as modified by Mitchell and Lunan (18). NaDodSO4 and other reagents for polyacrylamide gel electrophoresis came from Bio-Rad. All other chemicals were reagent grade.

Purification of HLA-A2 and -B7 Antigens. HLA antigens were purified from JY lymphoblastoid cell membranes as described (6). The only modification was to make a final exchange of 0.1% deoxycholate in 10 mM Tris (pH 8.0) before high-pH elution and neutralization from the antibody affinity columns. Purified HLA-A2 and -B7 antigens in 0.1% deoxycholate/10

Abbreviation: β2M, β2-microglobulin.
mM Tris, pH 8.0, were stored at −20°C until used.

Limited Proteolysis of HLA-A2 and -B7. 32P-Labeled HLA-A2 and HLA-B7 samples phosphorylated by pp60Src kinase in 0.1% deoxycholate/10 mM Tris, pH 8.0, were mixed with unlabeled carrier HLA-A2 or HLA-B7 in the same buffer. Each sample subjected to proteolysis contained 10 µg of unlabeled carrier HLA-A or -B, and final weight ratios of HLA to trypsin were 0, 1:1, 0.001, 1:500, 1:50, and 1:10 in a final volume of 100 µl of the above buffer. Samples were incubated with protease for 30 min at 37°C. The reaction was stopped by the addition of 1 µl of 100 mM phenylmethanesulfonyl fluoride, and the samples were placed on ice for 5 min. The samples were precipitated and then prepared for NaDodSO4/polyacrylamide gel electrophoresis.

Phosphotransferase Reactions Using pp60src. Phosphotransferase reactions were carried out at 30°C for 10 min (the reaction is linear for 30 min) in a total volume of 30 µl in the presence of 20 mM ATP, 10 mM MgCl2, and 10 mM Tris at pH 7.5. The amount of each substrate was approximately 200 ng per reaction, and the amount of pp60src was about 5 ng per reaction. The products were resolved by polyacrylamide gel electrophoresis and localized by autoradiography, and the radioactivity quantified by liquid spectrometry.

Analytical Methods. Electrophoresis on NaDodSO4/polyacrylamide on slab gels was performed according to the method of Laemmli (19). Either a 10% continuous acrylamide gel in a 5-cm-long running gel or a 7–15% gradient of acrylamide in a 30-cm-long running gel was used. Autoradiography was performed on wet or dry gels by using Kodak XAR-5 or SB-5 x-ray film, with and without enhancing screens.

Phosphorylated amino acid residues were identified by cutting heavy chains of HLA from an unfixed wet gel and eluting the protein into 1% deoxycholate/10 mM Tris, pH 8.0, and preparing this sample for high-voltage paper electrophoresis. The eluted protein was precipitated with 10% trichloroacetic acid and then washed with acetone to remove excess acid. After the residue was dried under nitrogen, 50 µl of concentrated ammonium hydroxide was added and the mixture was then vacuum dried; 50 µl of 6 M HCl was then added, and the sample was vacuum sealed and heated at 112°C for 1.5 hr. The sealed tube was cooled, and the HCl was removed by lyophilization. The final residue was suspended in 10 µl of pH 3.5 buffer [pyridine/acetate/H2O; 1:10:189 (vol/vol)]. The sample and phosphoamino acid standards were then spotted on 3M Whatman paper and electrophoresed in one dimension with pH 3.5 buffer at 3,000 V for 50 min. These conditions cleanly resolve phosphoserine, phosphothreonine, and phosphotyrosine. Electrophoresed samples were exposed on XAR-5 film with enhancing screens.

Computer Analysis. The DNA sequences of human and murine major histocompatibility complex class I genes were compared by using a homology matrix program (20). This program scores sequence homology over an adjustable range of nucleotide sequence and prints a score of percentage relatedness for that region. The flexibility of this program permits comparison of distantly related sequences with a high filtering of noise.

RESULTS

HLA-A2 and -B7 Antigens Are Phosphorylated by pp60src in Vitro. When purified detergent-soluble HLA-A2 or -B7 antigens were incubated in the presence of purified pp60src and [γ-32P]ATP, the HLA antigens were found to be labeled with phosphate as shown by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 1). The 44,000-dalton HLA heavy chains of both HLA-A2 and -B7 were labeled but no label was found associated with the 12,000-dalton β2M subunits under these conditions. The sarcoma kinase was also labeled during the incubation; autophosphorylation of this kinase is normally observed in vitro (9).

Several proteins have been shown to be substrates for pp60src (21). The rate of phosphorylation (in µmol/min per mg) in the present study were: HLA-A2, 2.8; HLA-B7, 10.3; tubulin, 24.7; 34-kd protein, 0.08; casein, 46.4; and pp60src (autophosphorylated), 5.2. These HLA preparations were free of endogenous protein kinases. No phosphorylation was detected in the absence of added enzyme and, as shown below, only a single tyrosine was phosphorylated. The HLA antigens are relatively good substrates in their undenatured form. In contrast, there are multiple phosphorylation sites in both casein and denatured tubulin as judged from phosphopeptide digestion patterns (data not shown). Also, a wide range of reaction conditions was not tested, and these rates apply only to the conditions examined. The significance of these rates may also be judged by the phosphorylation of the 34,000-dalton protein that is apparently a natural substrate of pp60src in transformed cells (22) and, under these conditions, is a poor substrate for pp60src.

Site of Phosphorylation in the COOH Terminus. Limited proteolysis of the labeled HLA-A2 and -B7 antigens was performed to localize the site of phosphorylation on the heavy chain. Trypsin is known to release COOH-terminal peptides of the 44,000-dalton heavy chains of HLA-A and -B, resulting in the generation of a 39,000-dalton chain (23). When pp60src-catalyzed 32P-labeled HLA-A2 or -B7 antigen was incubated with increasing amounts of trypsin, the 44,000-dalton heavy chain was converted to a 39,000-dalton species (Fig. 2A). The proteolysis of the heavy chain to 39,000 daltons was coincident with the loss of radioactive label (Fig. 2B). When HLA-A2 and -B7 antigens are 32P-labeled in vivo and subsequently extracted, purified, and trypsinized, the 44,000-dalton heavy chain loses its radioactive COOH terminus and is converted to a 39,000-dalton nonradioactive polypeptide chain (2). Trypsin liberated the bound phosphate of pp60src-labeled HLA-A2 and -B7 as the molecules lost their COOH-terminal peptides; therefore, the site of phosphorylation is in the COOH terminus.

Amino Acid Phosphorylated in the COOH Terminus Is Tyrosine. High-voltage paper electrophoresis was used to analyze acid-hydrlyzed 32P-labeled HLA-A2 and -B7 heavy chains to determine the identity of the phosphoamino acid labeled by pp60src. The autoradiographs obtained revealed phospho-
rosine as the exclusive $^{32}P$-labeled phosphoamino acid of the HLA-A2 and -B7 heavy chains (Fig. 3). The site of the labeled phosphate residue could be determined using both the protein (13) and DNA (24) COOH-terminal sequences for HLA-A2 and -B7 heavy chains (Fig. 4). In fact, the COOH-terminal intracellular domains of these heavy chains contain only one tyrosine residue, at amino acid position 320. Thus, both trypsin localization and amino acid identification by high-voltage electrophoresis identify the site of pp60$^{v-src}$ labeling as the single tyrosine residue of the COOH-terminal intracellular domain of HLA-A2 and -B7 antigens.

Protein Sequences of the Intracellular Domains of Human and Murine Class I Antigens Include a Highly Conserved, Tyrosine-Containing Amino Acid Segment. The in vitro tyrosine phosphorylation of HLA-A and -B by pp60$^{v-src}$ prompted examination of other human and murine class I intracellular domain protein sequences and protein sequences deduced from either cDNA or genomic clones (13, 25–31). The aligned protein sequences for known human and murine intracellular domains are presented in Fig. 4. The intraspecies homology for

**FIG. 3.** One-dimensional high-voltage paper electrophoresis of acid-hydrolyzed HLA-A2 (lane 1) and HLA-B7 (lane 2) antigens $^{32}P$-labeled by pp60$^{v-src}$. Samples were run in pH 3.5 buffer. Phosphotyrosine is the exclusive phosphoamino acid residue.

**FIG. 4.** Amino acid sequences for the intracellular domain of human and murine class I histocompatibility antigens as determined by protein sequence determination methods or as deduced from DNA sequence information. Sequence information was gathered from the following sources: pHLA-2 (HLA-B7) (25), HLA-A2 (13), pHLA12.4 (26), H-2K$^b$ (27), H-2L$^d$ (28), pH-2$^d$-1 and pH-2$^d$-3 (29), pH-2$^d$-4 (30), and gene 27.1 (31). pHLA-2 is our nomenclature for the unnamed plasmid described in ref. 25. The human intracellular domain sequence pHLA-2 was used for alignment of all other sequences. The human sequences HLA-A2 and pHLA12.4 line up at the identical amino acid positions; the remaining murine sequences are aligned by a shift of one amino acid. — — Homology with pHLA-2; a letter represents a change from the pHLA-2 sequence. Notice the conservation of tyrosine (boxed Y) across all sequences except in the murine pseudogene 27.1. Arrowheads denote exon boundaries as determined by DNA sequence analysis.
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FIG. 5. Genomic organization of human and murine class I genes. The arrangement of exons as presented by Malissen et al. (26) for HLA and by Steinmetz et al. (31) for H-2 are depicted to show which exons code for the intracellular domains. Three exons in the human and four exons in the mouse are required for the composite intracellular domain.

human and murine sequences is high. The overall homology between species is not striking for this domain. However, there is a highly conserved segment of amino acids which contains a tyrosine residue at position 320 in the human sequence and position 321 in the mouse. Starting with amino acid 316 in human and 317 in murine sequences, the segment -K-G-G-X-Y- is 100% conserved in seven of eight sequences for expressed products. The exception is gene pH-2d-4 which deviates by two amino acids. Beyond this brief segment of amino acids, the overall homology between species is low. However, at positions 313, 326, 328, and 332 serines and threonines are either conserved or used as replacements for each other.

Multiple serine and threonine residues are scattered throughout the intracellular domains of class I antigens. Despite the preponderance of these two phosphorylatable residues, only one tyrosine residue is present in seven of eight sequences, and that tyrosine is present in the identical position for all sequences examined. The only other tyrosine present in the eight expressed sequences is found in clone pH-2d-4 at amino acid position 327 where it appears instead of a serine present in all other sequences. In the H-2 pseudogene 27.1 the tyrosine is replaced by cysteine at position 321; this gene is not expressed in the mouse. The positioning and conservation of tyrosine 320/321 in the human and murine intracellular domains suggests that this tyrosine may be used in some cellular function.

Human and Murine Class I Genes Contain a Highly Conserved Tyrosine Encoding Exon. The intron/exon organization of HLA gene pHLa12.4 (26) and the gene organization of H-2 pseudogene 27.1 (31) are presented in Fig. 5. The region of DNA encoding the intracellular domain is highlighted to demonstrate the use of three exons in the human and four exons in the mouse to encode an overall polypeptide segment of only 32 amino acids or, in the case of H-2Kb and pH-2d-4, a segment of 41 amino acids.

Table 1. Computer analysis of homology among the DNA sequences for the intracellular domain of class I human and murine DNA sequences

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<th>Clone</th>
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<th>23-55</th>
<th>56-96</th>
<th>1-96</th>
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Sequences were compared by homology matrix analysis. Clone pHLa2 was compared with all other sequences along the 96-nucleotide length. The sequences were also compared for the 3' end of exon 5 (nucleotides 1-22), exon 6 (nucleotides 23-55), and exon 7 (nucleotides 56-96). The first 96 nucleotides of the total 123 nucleotides available in this region for H-2Kb were used for comparison with all other sequences. The overall homology from nucleotides 1-96 was examined as was the homology in individual exons. Numbers presented are scores of percentage identity between two individual sequences.

The DNA sequences deduced as encoding human and murine intracellular domains were aligned with pHLa2 (a cDNA clone encoding HLA-B7) by use of exon boundaries (Fig. 6). This alignment of the DNA results in the identical alignment shown for the protein sequences in Fig. 4. The nucleotide sequence was numbered by using the first nucleotide for the donor of the first amino acid of the intracellular domain of pHLa2 (methionine) and ends in nucleotide 96 encoding the final amino acid (alanine). The other human and all murine sequences were compared to pHLa2 (Table 1). Overall intracellular domain homology (nucleotides 1-96) between the two human sequences pHLa2 and pHLa12.4 is 89%, whereas the overall homology between pHLa2 and all the individual murine sequences is on the order of 50%. When the 3' end of exon 5, exon 6, and exon 7 of pHLa2 are compared to the analogous exons of pHLa12.4, each exon displays approximately 90% homology. When the separate exons of pHLa2 are compared to the separate analogous exons on the murine sequences, a striking homology between human and mouse at exon 6 becomes apparent. This tyrosine-encoding exon 6 is also 75% homologous across species; the nucleotides in the 3' end of exon 5 show approximately 64% homology, and in exon 7 homology is only 38%.

In the murine system the homologies between individual analogous exons are high, and it is particularly noteworthy to find 100% conservation in exon 6 among sequences H-2Kb, H-2Lb, pH-2d-1, and pH-2d-3. The physical isolation and conservation of exon 6 suggests that there has been strong selective pressure to preserve this segment of DNA and the tyrosine-containing protein sequence encoded there.

FIG. 6. DNA sequences for the intracellular domain of class I human and murine histocompatibility antigens. The arrangement of codons is identical to the amino acid alignments in Fig. 4. The DNA sequences are gathered from the sources cited in Fig. 4. The human DNA sequence pHLa2 is used for comparison of all other DNA sequence comparisons. Homology with pHLa2; a letter shows a change at that nucleotide position. Solid vertical lines denote exon boundaries. Notice the high degree of homology in exon 6 across species.
DISCUSSION

Tyrosine-320, which is labeled by pp60^Src in both HLA-A2 and -B7, is part of a conserved protein sequence -K-G-G-S-Y-. This protein sequence is not similar to a sequence published for in vivo and in vitro phosphorylation sites of pp60^Src in which a number of acidic residues were located NH2-terminally to the 32P-labeled tyrosine (32). However, those authors also noted that, in vertebrate cells, pp60^Src is apparently labeled in vivo at a different and unidentified tyrosine residue. The sequence presented in this paper may define parameters to consider in pp60^Src substrate specificity or it may simply indicate that this kinase demonstrates a relaxed substrate specificity in vitro.

We have examined an exogenous phosphorylation event by using an avian virus kinase to label a human cell surface antigen. A more natural kinase-substrate relationship might be found by examining the in vivo effect of pp60^Src on chicken B complex (major histocompatibility complex equivalent) class I antigens, should these molecules possess a tyrosine residue as part of their intracellular domain. Ultimately it would be useful to know whether the vertebrate homologue pp60^Src will phosphorylate HLA antigens in vivo. Several other cell surface proteins have recently been shown to contain phosphotyrosine residues, i.e., the receptors for insulin (33) and epidermal growth factor (34) are phosphorylated at tyrosine residues by appropriate hormone stimulation. Platelet-deprived growth factor and epidermal growth factor increase the amount of phosphotyrosine in 42,000- and 45,000-dalton proteins of NIH 3T3 cells (35). Murine class I heavy chains are in this size range.

There are up to nine serines and one threonine residue (which is 30% of the total amino acids) in the intracellular domains of the individual human sequences, but only one tyrosine residue is present. Recently it has been determined that pp60^Src is associated with cell membranes (12). This fact adds credence to the idea that transmembrane proteins such as HLA and H-2 may be candidates for phosphorylation at tyrosine by endogenous sarcoma kinase homologues. In this case the juxtaposition of kinase and substrate would be localized to the plane of the cytoplasmic face of the cell membrane.

Gilbert has proposed that exons represent domains in the sense of structural and functional elements contributed to proteins (36). If this concept is applied to the exons encoding the intracellular domain of HLA, then it is conceivable that exon 6 might encode a functional element distinct from that for exon 7, and each could serve separate functional requirements within the cell. The intracellular domains of HLA and H-2 antigens are known to be phosphorylated at serine(s) in vivo. In all the mouse sequences, the only serine residues present intracellularly are those encoded by exons 5 and 6 (with positions 326, 328, and 332 being highly conserved) and the only residue with the potential for being phosphorylated in exon 6 is the single tyrosine residue. Perhaps in different physiological situations the position of the segment that is phosphorylated may change. Could the loss of the tyrosine residue in the H-2 pseudogene 27.1 have eventually contributed to the loss of expression of this gene or were the termination codons the immediate cause?

Examination of the highly conserved tyrosine residue in the intracellular domain of HLA and H-2 may help to elucidate intracellular domain functions. Mutagenesis of the tyrosine codon in class I gene clones and expression of those genes in transfected cells may provide the opportunity to study the function of this residue.

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