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Immunology

Human homologue of murine tumor rejection antigen gp96: 5′-Regulatory and coding regions and relationship to stress-induced proteins

(tumor immunity/heat shock protein/glucose-regulated protein)

ROBERT G. MAKI*†, LLOYD J. OLD‡, AND PRAMOD K. SRIVASTAVA*§

*Box 1215, Department of Pharmacology, Mount Sinai School of Medicine, City University of New York, New York, NY 10029-6574; and ‡Sloan–Kettering Institute for Cancer Research, New York, NY 10021

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ABSTRACT **Cell-surface glycoproteins of 96 kDa (gp96) have been implicated in immunogenicity of methylcholanthrene-induced mouse sarcomas in syngeneic hosts. In view of the potential immunogenicity of gp96-related molecules in human tumors, we have defined the 5′-regulatory and complete coding regions of a human gp96 transcript. The 5′-regulatory region contains an imperfect heat shock element apart from other regulatory sequences. The amino acid sequence of human gp96 is 96% homologous to its murine counterpart and genes for the two molecules show significant homology between untranslated regions. Comparison of gp96 sequences to other sequences in DNA and protein data bases indicates significant homology with the stress proteins 94-kDa glucose-regulated protein (grp94) and 108-kDa heat shock protein (hsp108) and 99-kDa endoplasmic reticular protein (ERp99). These molecules are either identical or represent a family of closely related molecules. With regard to their role in tumor immunity, it needs to be determined whether gp96 molecules are tumor antigens *per se* or whether they serve as carriers of other immunogenic moieties.**

Structural identification of tumor antigens that can elicit tumor rejection in the host of origin is the central aim of tumor immunology. In animal systems, the immunogenicity of such antigens can be monitored *in vivo* by tumor transplantation assays, and progress has been made in defining antigens that elicit transplantation immunity in several mouse and rat tumors (1–3). In contrast, immunological analysis of human cancer is limited in the main to the study of humoral and cellular antitumor responses that can be demonstrated *in vitro*. Melanoma has been extensively studied in this regard and antibodies with apparent specificity for autologous melanoma cells have been found in a small proportion of patients with melanoma (4). Specific T-cell responses to melanoma cells have also been reported by a number of laboratories using interleukin 2-dependent T-cell cloning (5–10). One of the serologically defined human tumor antigens has been partially characterized (11), but identification of T-cell-defined human melanoma antigens has remained elusive. Boon and his colleagues (12) have described an approach for identifying T-cell recognized epitopes on mouse tumors. This method, involving transfection and molecular cloning, shows much promise for the comparable analysis of human tumor antigens defined by T cells.

Immunogenicity of chemically induced sarcomas of inbred mice has received the greatest attention in experimental systems. These tumors elicit immunity to large challenges and display extensive diversity—i.e., each tumor in a given mouse strain appears to have its own individually distinct antigenicity. We have previously shown that 96-kDa cell-surface glycoproteins (gp96) derived from two antigenically distinct BALB/c mouse sarcomas (Meth A and CMS5) elicit resistance to tumor transplants; Meth A gp96 elicits resistance to Meth A but not CMS5, and CMS5 gp96 elicits resistance to CMS5 but not Meth A (13). cDNA clones encoding gp96 from these chemically induced tumors and from mouse spleen have been isolated and sequenced (1, 14). No structural changes have been detected in tumor-derived gp96. Rabbit antisera to murine gp96 detect cross-reactive gp96 molecules in human and rat tissues, indicating that gp96-related molecules are widely distributed and phylogenetically conserved (ref. 15; unpublished data). In the present study, the human counterpart of murine gp96 has been defined by cDNA and genomic cloning.¶

MATERIALS AND METHODS

DNA and RNA Extraction: Southern and Northern Blot Analysis. Genomic DNA preparations from human tumor cell lines, total cellular RNA and poly(A)$^+$ RNA isolation, and Northern and Southern blotting were performed as described (16). RNA probes were prepared by T3 or T7 promoter-driven transcription in plasmid pT3-T7-18 or pT3-T7-19 (Life Technologies, Bethesda, MD).

cDNA Library Construction and Screening. The library LASTD2 was constructed from human teratocarcinoma cDNA in the vector Charon BS(−) (17). DNA from positive clones was digested with the restriction enzyme *Not* I, heat inactivated, diluted 1:5 with water, and buffer (final concentrations, 50 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/1 mM ATP/1 mM dithiothreitol/5% polyethylene glycol 8000) and 1 Weiss unit of T4 DNA ligase were added directly. Aliquots of this mixture were used to transform *Escherichia coli* strains LE392 or DH5α.

Sequencing. Sequencing was performed on small-scale preparations of plasmid DNA or on single-stranded DNA derived from R408 helper phage superinfection (18) of plasmid-bearing *E. coli* NM522 by dideoxynucleotide sequencing using modified T7 DNA polymerase or *Thermus aquaticus* DNA polymerase according to the manufacturers' instructions. In addition, a partial set of deletion subclones was

Abbreviations: gp96, 96-kDa glycoprotein; hsp, heat shock protein; grp, glucose-regulated protein; ERp99, 99-kDa endoplasmic reticular protein; nt, nucleotide(s).

†On leave of absence from: Immunology Program, Sloan–Kettering Division, Cornell University Graduate School of Medical Sciences, New York, NY 10021.
§To whom reprint requests should be addressed.

¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M24747 and M33716 for the human gp96 cDNA and the gp96 promoter, respectively).

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constructed for sequencing from both the 5′ and 3′ ends of the clone by the *Exo* III/mung bean nuclease method (19).

Primer-Extension Analysis. Primer-extension reactions using 40,000 cpm of end-labeled oligonucleotide were performed with yeast tRNA, total cytoplasmic human RNA, and poly(A)$^+$ human RNA as described (16).

RESULTS

Derivation and Characterization of gp96 cDNA Clones. Approximately 1,000,000 clones from cDNA library LASTD2 were probed with the murine gp96 cDNA probe λsex6. Nineteen positive phagemid clones were purified; two phagemids with the largest inserts were subcloned, yielding the plasmids pH48 and pH52. Fig. 1 is a composite derived from pH48 and pH52 and indicates an open reading frame encoding a potential 803-amino acid 92,450-Da protein with a predicted pI of 4.54. Hydropathicity analysis of pH48, averaging over a six-residue window (21), demonstrates an 18- to 19-residue N-terminal hydrophobic region, presumably encoding a signal peptide. Methods predicting signal peptide cleavage (22) indicate that the two most likely sites for cleavage lie after the 17th and 21st residues. The human gp96 signal peptide is therefore provisionally defined as containing 21 amino acids, corresponding to the signal peptide cleavage site in mouse gp96 (14). The mature 782-amino acid protein has a predicted molecular mass of 90,170 Da.

pH48/52 contains a 5′ untranslated region [nucleotides (nt) 13–105], translation initiation codon ATG (nt 106–108), signal peptide (nt 106–168), five potential N-linked glycosylation sites (starting at nt 424, 754, 1438, 1546, and 1609), a TAA stop codon (nt 2515–2517), 3′ untranslated region (nt 2518–2780), polyadenylylation signal (nt 2762–2767), and a 12-base-pair (bp) poly(A) tail (attached to nt 2780). A sequence putatively involved in destabilizing mRNA (23) is found twice in the 3′ untranslated region (starting at nt 2563 and 2717). The amino acid sequence KDEL is located at the C terminus of the open reading frame.

gp96 Transcripts in Human Tumor Cells. Different amounts of gp96 transcripts are present in various amounts in a range of human tumor cell lines. The human gp96 transcript is ≈2.8 kilobases (kb) long, which is also the size of the mouse gp96 transcript. In a series of >30 cell lines of various histological origins, no tumor cell line was found lacking gp96 transcripts.

Southern Blot Analysis of Human Genomic DNA with gp96 cDNA pH48. Human genomic DNA derived from tumor cell lines was digested with several restriction enzymes and probed with fragments derived from pH48. As shown in Fig. 2, digests of DNA from neuroblastoma cell line LA-1-15n with seven restriction enzymes yielded three or four hybridizing bands using 5′ or 3′ probes from pH48. Multiple hybridizing bands were also observed with genomic DNA derived from other human cell lines. These data suggest that there are three or four gp96-related genes per haploid human genome. Four tumor-derived cell lines and two peripheral blood lymphocyte specimens were tested by Southern blot analysis; no gross differences in restriction pattern with seven restriction enzymes were observed with 5′ and 3′ pH48 probes.

Analysis of gp96 Transcription Start Site. Fig. 3 represents a primer-extension analysis of HeLa mRNA with an antisense oligonucleotide (nt 58–93 of the sequence in Fig. 1).

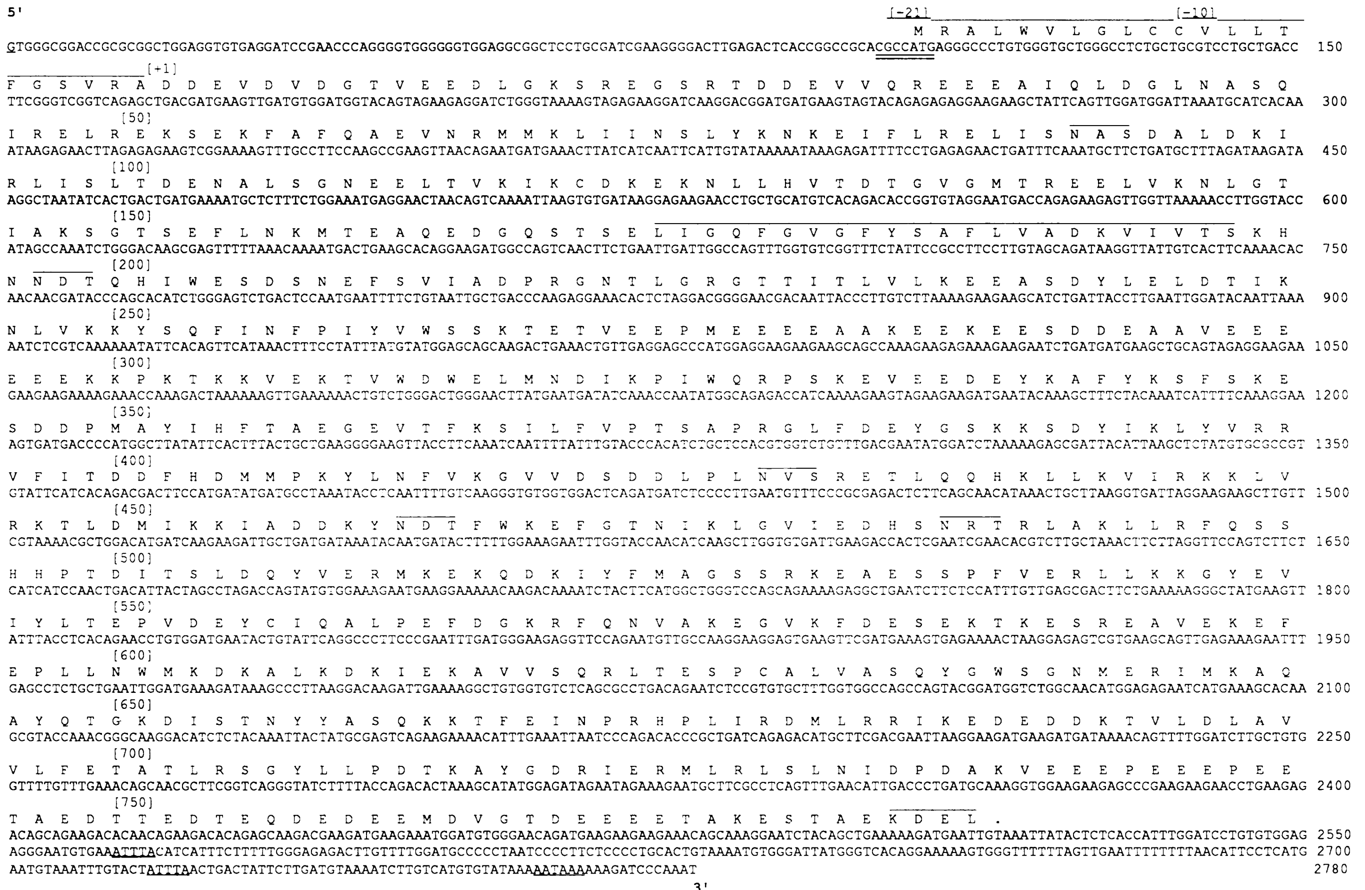

FIG. 1. Complete sequence of the human gp96 transcript derived from pR8 (nt 1–12) and pH48/52 (nt 13–2780). Residue +1 is the site of transcription initiation. Other features include ribosomal binding site/translation start site (20) (double underlined), 21-amino acid signal peptide (overlined), N-linked glycosylation sites Asn-Xaa-Ser/Thr (NXS/T, overlined), potential transmembrane region (overlined), Lys-Asp-Glu-Leu endoplasmic reticular retention sequence (KDEL, overlined), ATTTA mRNA destabilization signals (underlined), and AATAAA poly(A) addition signal (underlined). A 12-residue poly(A) tail (not shown) is found after nt 2780.

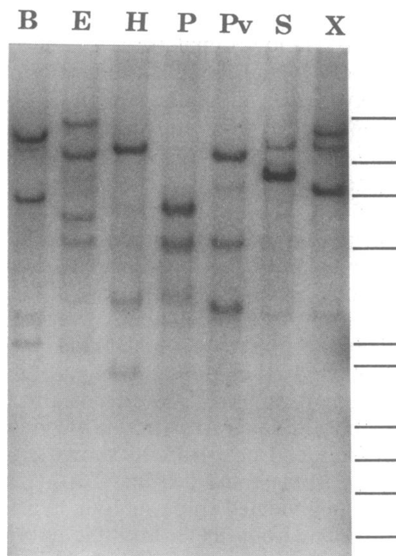

FIG. 2. Southern blot analysis of human neuroblastoma cell line LA-1-15n genomic DNA. Fifteen micrograms of total genomic DNA was digested with the indicated restriction enzymes and electrophoresed over 0.8% agarose, blotted, and hybridized 16 hr to random ^{32}P-labeled DNA probe pH48ΔG (3' 314 bp of pH48 cDNA) at 60°C. An autoradiogram exposed for 3 days with two intensifying screens is shown. Molecular size markers are indicated as bars to the right (top to bottom): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.35, 1.1, 0.9, and 0.6 kb. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* II; S, *Sst* I; X, *Xba* I.

The major visible band indicates that the mature mRNA is initiated 57 bp from the primer 3' terminus. This places the start of transcription 12 bp upstream of the first base of the cDNA clone pH48. The sequence of this region was determined by analysis of a genomic clone pR8, discussed below, which represents the 5' end of the coding gp96 gene.

Sequencing of gp96 Genomic Clone pR8. Genomic clones hybridizing to a probe for the 5' end of pH48 were isolated from a peripheral blood lymphocyte DNA library. One genomic clone was digested with *Eco*RI and subcloned into pBluescriptII KS(−). Subclone pR8 contained the 5' coding region of pH48 and its sequence was determined by using the oligonucleotide (nt 58–93) used for primer extension. One CCAAT box (nt −195), five inverted CCAAT boxes (nt −300, −220, −141, −107, and −76), and one consensus Ap2 site (nt −442) (Fig. 4) are observed upstream of the transcription start site. In addition, a consensus site for one of several factors that bind to the E4 promoter of adenovirus (E4TFI) is found at nt −41. Five consensus Spl sites (nt −571, −272, −258, −129, −50) are evident within the 800 bases upstream of the transcription start site, and an additional Spl site is observed in the 5' untranslated region at nt +3. One imperfect heat shock transcriptional control element (HSE) is located upstream of the start of transcription (nt −118), showing a 6/8-bp match with consensus sequence CNNGAANNTTCNNG. Another weak HSE is seen in intron I (nt 206). A TATA box is observed 32 bases upstream of the start of transcription, but it differs significantly in sequence from the consensus TATAAA.

The putative gp96 promoter and first exon are rich in G and C nucleotides (59.8%) and CpG dinucleotides; the ratio of CpG to GpC dinucleotides in this region is 0.94, defining the region immediately 5' to the start of transcription as a CpG "island" (24). In addition, several Spl sites are observed in this region. These structural features characterize other constitutively expressed genes (25, 26).

Comparison of Murine and Human gp96: Homology with Stress-Induced Proteins. Human and murine gp96 show 96% homology at the amino acid level and 90% homology at the DNA sequence level. Of the 25 differences in the amino acid sequences, 18 (72%) are conservative changes. Similarly, of the 190 differences at the nucleotide level, 161 (85%) occur in the third base of a codon. In contrast to the strong conservation of coding regions, the first four introns of the two genes show much less homology (unpublished data). In relationship to other highly conserved proteins, mouse and human gp96 have a higher degree of homology than α-hemoglobin (80%), histone H3 (87%), or cytochrome *c* (91%), although they are less conserved than α-tubulin (>99%) or skeletal muscle actin (100%).

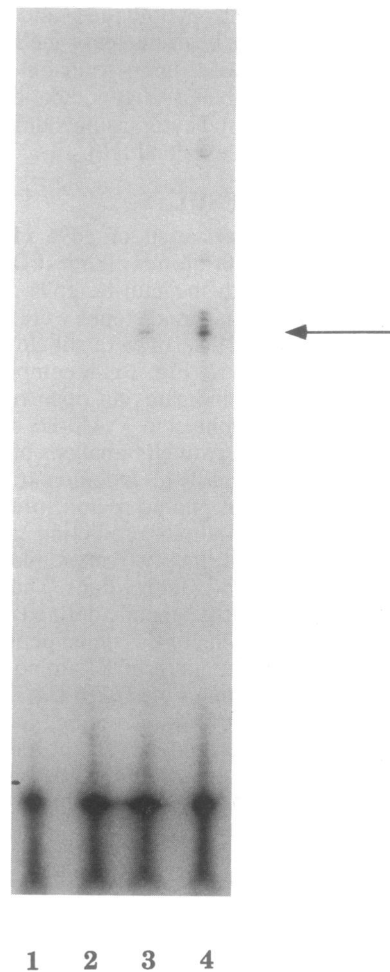

FIG. 3. Primer-extension analysis of HeLa RNA using 36-mer antisense oligonucleotide (nt 58–93) of Fig. 1. γ-^{32}P-end-labeled oligonucleotide (40,000 cpm) was hybridized overnight to 10 μg of yeast tRNA (lane 2), total cytoplasmic HeLa RNA (lane 3), twice enriched poly(A)$^{+}$ HeLa RNA (lane 4), or not hybridized (lane 1) before primer extension and electrophoresis on a 6% polyacrylamide sequencing gel. The major extension product (arrow) is observed 57 bases from the end of the primer, although other species are also visible.

Comparison of gp96 with other sequences in the data bases shows homologies with stress-induced or heat shock proteins hsp100 (27) and hsp90 (28). At the amino acid level, >90% homology is observed between gp96 and hsp100, and 50% between gp96 and hsp90. In addition to homologies in the coding region, homologous regions are also observed between human gp96, grp94 (glucose-regulated protein), and chicken hsp108 promoters (ref. 29; Fig. 4). There is also a homology, noted previously, with the endoplasmic reticular protein ERp99 (30). Coding regions of hsp70 and other stress proteins do not show significant homology with gp96.

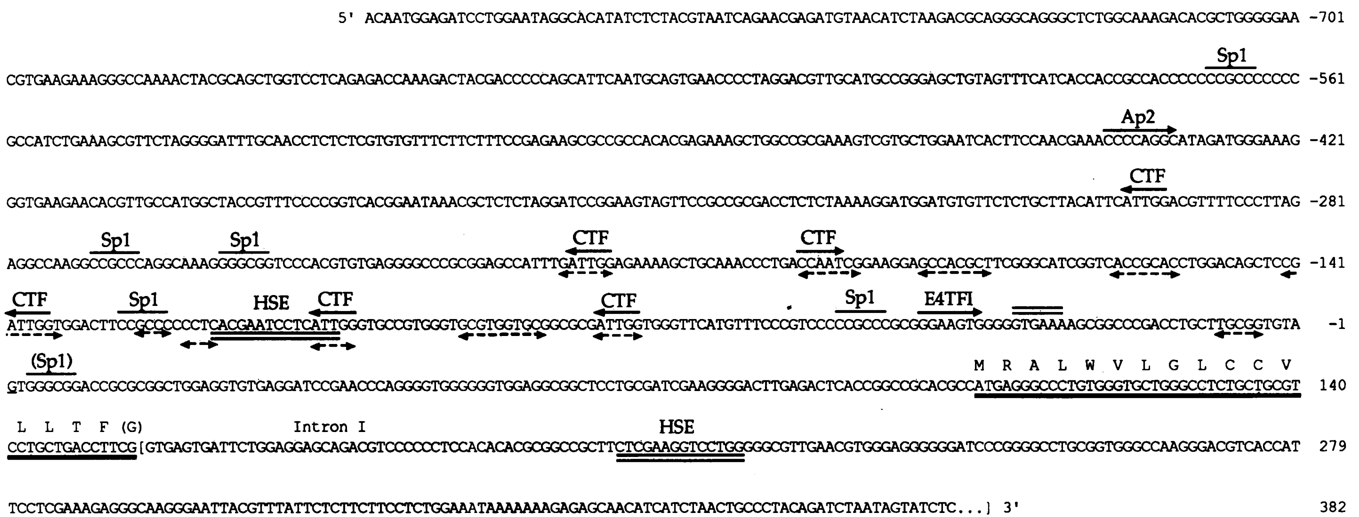

FIG. 4. Partial sequence of genomic clone pR8 encoding the 5′ region of a human gp96 gene and promoter. Features include possible transcription factor binding sites (overlined with name of factor), transcription start site (base +1, underlined), imperfect heat shock element (HSE) (nt −118 to −105 and nt 206–219, double underlined), atypical TATA box (double overline), translation start site and exon 1 (heavy underline), 5′ region of intron I (in brackets), and conserved regions between chicken hsp108, human grp94, and human gp96 (dashed underline with double-headed arrows).

DISCUSSION

Several gp96-related genes have now been found in both mice and humans. Short probes derived from different regions of human gp96 cDNA hybridize with as many as four bands on Southern bands of human genomic DNA digested with a number of restriction enzymes, and human gp96 genes map to at least two different chromosomes (unpublished data). gp96 cDNA clones derived from a human tumor have been used to analyze one of these genes and its associated transcript.

Organization of the 5′-regulatory region of human gp96 provides some clues to the apparently constitutive nature of gp96 expression. Housekeeping genes are generally associated with unmethylated CpG islands encompassing the site of transcription initiation. CpG islands can be activated regardless of their site of methylation by some transcription factors (e.g., Sp1; ref. 31) and lack of CpG methylation is presumably maintained by binding of such factors. The 5′-regulatory region and exon 1 of human gp96 represent a CpG island containing six Sp1 binding sites. The atypical TATA box of human gp96 and heterogeneity of transcription initiation as determined by primer extension are common features in other housekeeping genes (25, 26). The 5′ upstream region contains several other consensus transcriptional factor binding sites, including a CCAAT box, five inverted CCAAT boxes, and one Ap2 site; the CCAAT box is not generally observed in promoters of constitutively expressed genes. Structure and function of a similar promoter (human grp94) have been demonstrated by Chang *et al.* (29).

The longest open reading frame of the human gp96 transcript contains a 5′ signal peptide-encoding region and five potential N-linked glycosylation sites. At the 3′ end of the coding region, a sequence encoding the tetrapeptide Lys-Asp-Glu-Leu (KDEL) is observed. This sequence has been found in proteins localized to the endoplasmic reticulum (32–34). Murine ERp99, a protein that closely resembles gp96, also has a C-terminal KDEL sequence (30) and is a major component of the endoplasmic reticulum. Despite the presence of a C-terminal KDEL sequence, the presence of gp96 on the cell surface has been demonstrated by a number of criteria: (*i*) immunoprecipitation of gp96 from surface radioiodinated tumor cells; (*ii*) detection of gp96 on the cell surface by erythrocyte rosetting on live cells; and (*iii*) isolation of gp96 from purified plasma membranes (13). Presumably, a fraction of gp96 makes its way to the cell surface either through the continuity of endoplasmic reticulum membrane with the plasma membrane or by a specific mechanism that modulates the influence of the KDEL sequence. Some normally secreted proteins—e.g., rat growth hormone and α subunit of chorionic gonadotropin—are not retained in the endoplasmic reticulum in spite of the addition of a C-terminal KDEL sequence, supporting the notion that KDEL-mediated endoplasmic reticular retention of gp96 is not absolute (35).

The 5′ untranslated, coding, and 3′ untranslated regions of murine and human gp96, grp94 (29), mouse ERp99/endoplasmin (30), and chicken hsp108 (27) show a high degree of homology. These observations indicate that gp96, grp94, and ERp99 are identical or belong to an extended family of closely related molecules. The sequence of the human grp94 promoter has been published (29) and is identical to the sequence of the human gp96 promoter region except for differences in 11 G and C residues, indicating that gp96 and grp94 are either distinct genes or alleles of the same gene. The complete sequence of the coding region of human grp94 has not been published; hence, the precise degree of homology between this gene and gp96 is not known. A cDNA clone for a human hsp108, a glycoprotein biochemically indistinguishable from grp94, has recently been reported (36). Human hsp108 is 97% (nucleotide level) and 94% (amino acid level) homologous to chicken hsp108. In contrast, human gp96 is 81% (nucleotide level) and 89% (amino acid level) homologous to chicken hsp108. In view of the fact that complete sequence data on human hsp108 are unavailable, it is difficult to formally resolve whether these proteins are identical, closely related, or represent different alleles.

The possibility that stress-induced proteins play an important role in immune response in infectious and other diseases has recently attracted considerable attention. Humoral and cellular responses to several stress proteins have been detected in animals and humans infected with bacterial and parasitic agents (37, 38). In most cases, origin of the immunogenic stimulus has not been defined: is the immune response elicited by stress proteins of the infectious agent or the host? Because of the highly conserved nature of stress proteins (39), this is not an easy question to answer. Another unresolved issue is the role of stress proteins in disease

development: is the immunity to stress proteins protective, injurious, or merely a by-product of the host immune response to disease? These questions are also relevant to finding antibodies and/or T cells to stress proteins in patients with immune diseases such as rheumatoid arthritis (37, 38).

With regard to immune response to cancer, the work of Ullrich *et al.* (40) raised the possibility that heat shock proteins may be recognized as tumor antigens eliciting protective immunity. These investigators isolated 84/86-kDa molecules from chemically induced tumors that induce specific resistance to tumor challenge. The p84/86 antigens belong to the hsp90 family (28, 40), rather than the hsp100 family as in the case of gp96 molecules (this paper). In addition, immunity against murine melanomas (41) and a rat histiocytoma (G. Deshpandé and A. Khar, personal communication) can be elicited by serum albumin-like proteins; while albumin is expressed constitutively in adult life, it is inducible by heat shock in fetal tissues (42). Furthermore, immunogenicity of Ha-*ras*-transfected fetal rat fibroblasts has been correlated with the presence on the cell surface of a hsp70-related protein (43).

A key question is whether gp96 and these other stress protein-related antigens are tumor antigens *per se* or whether they serve as carriers for other immunogenic moieties. If, in fact, the tumor-specific immunogenicity of gp96 and p84/86 resides in the primary structure, then point mutations, deletions, or insertions could account for the extensive diversity of chemically induced tumors. Consequent changes in the encoded gp96 polypeptide could be recognized as antigenic determinants following normal antigen presentation pathways. If, on the other hand, gp96 or other stress proteins serve as carriers or presenting molecules for other immunogenic moieties, the association of peptides with class I and class II antigens of the major histocompatibility complex is a clear precedent (44–46). The ability of stress proteins to bind to a number of different molecules is consistent with this possibility (see ref. 47).

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