Molecular and Immunological Evaluation of the Transcription Factor SOX-4 as a Lung Tumor Vaccine Antigen

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The developmental transcription factor SOX-4 has been shown to be highly and differentially overexpressed in primary small cell lung carcinomas (SCLC). To examine the potential of SOX-4 for broad use as a lung cancer vaccine, we have evaluated the expression of SOX-4 in a panel of primary adenocarcinoma, squamous, and large cell tumor samples as well as in a panel of established small cell and non-small cell lung carcinoma tumor cell lines. SOX-4 mRNA is shown to be overexpressed in a substantial fraction of each of these lung tumor types. To examine the immunological potential of SOX-4, we have evaluated the presence of SOX-4-specific CD4 and CD8 T cells in PBMC of healthy donors and the presence of SOX4-specific Abs in sera from SCLC patients. We demonstrate the presence of both CD4 and CD8 T cells that recognize naturally processed epitopes derived from SOX-4 as well as the presence of SOX-4-specific Abs in sera from SCLC patients, but not in sera from healthy donors. The lung tumor-specific overexpression and demonstration of a comprehensive Ag-specific immune response specific for SOX-4 support the use of this molecule in the development of whole gene-, peptide-, or protein-based vaccination strategies against lung cancer. Furthermore, the identification of naturally processed T cell and Ab epitopes from SOX-4 provides valuable tools for the development of peptide-based vaccination strategies against lung cancer as well as to monitor SOX-4-specific responses in vaccinated patients. The Journal of Immunology, 2004, 172: 3319–3327.

Lung cancer is the leading cause of cancer deaths in the developed world, accounting for 30% of all cancer deaths (1). Long term prognosis for patients diagnosed with lung cancer is poor, with overall survival rates for non-small cell lung adenocarcinoma (NSCLC)5 and small cell lung carcinoma (SCLC) of 10–15% and <5%, respectively (2, 3).

Poor prognosis for survival of patients with lung cancer stems at least in part from the relative insensitivity of lung cancers to both radiotherapy and chemotherapeutic intervention (4). Thus, the development and application of new therapeutic strategies is essential for effective treatment of this disease. Vaccine-based immunotherapeutic strategies that activate a comprehensive anti-tumor immune response and result in activation of both the cellular and humoral arms of the immune system hold promise for the treatment of cancer. The development of effective vaccine-based immunotherapeutic strategies against lung cancer depends at least in part on the identification of appropriate Ags overexpressed by lung tumors, the presence of T cell precursors that recognize tumor cells that express the vaccine Ag, and the ability to deliver the vaccine in a manner that results in a comprehensive and effective Ag-specific immune response.

To date, relatively few molecules have been identified that could be considered vaccine candidates for the treatment of lung cancer (5–10). In most cases these molecules are expressed by either SCLC or NSCLC, but due to the differences in the cellular origin of these tumors, are not expressed by both lung cancer types. Furthermore, very little immunological data are available to validate the use of lung cancer candidates for vaccine-based immunotherapeutic strategies.

Using PCR-based subtraction approaches, we have recently described the identification and molecular characterization of a number of gene products overexpressed by primary small cell lung carcinomas (11). Among these genes were identified three members of the SOX gene family of transcription factors shown to be involved in embryological development. One of the SOX family members identified, SOX-4 (L978P), was shown to be highly and differentially expressed in a substantial fraction of SCLC samples and also in a pool of primary lung adenocarcinoma samples, with very low levels of expression in a number of normal essential tissues. Notably, evidence has been presented to suggest that SOX-4 may be involved in tumorigenesis (12, 13).

In this report we present molecular and immunological data to validate SOX-4 as a vaccine candidate for lung cancer. Quantitative real-time PCR analyses using SOX-4-specific primers demonstrate significant overexpression of SOX-4 in a variety of primary NSCLC samples, including adenocarcinoma and squamous and large cell lung tumors, as well as overexpression of SOX-4 in established SCLC and NSCLC tumor cell lines. Using in vitro stimulation protocols and PBMC derived from healthy donors, we demonstrate the presence of CD8+ and CD4+ T cell precursors that recognize naturally processed epitopes derived from SOX-4 and can specifically recognize lung tumors that express SOX-4. Finally, using sera from lung cancer patients, we demonstrate the presence of SOX-4-specific Ab responses in lung cancer patients.
MOLECULAR AND IMMUNOLOGICAL EVALUATION OF SOX-4

These results support the further development of SOX-4 as a vaccine Ag candidate with potential clinical utility for the treatment of both SCLC and NSCLC lung cancer.

Materials and Methods

Cells and cell lines

PBMC from normal individuals were isolated by apheresis, followed by separation on a Lymphoprep (Nycomed, Oslo, Norway) Ficoll density gradient. The review of the research protocol by the institutional review board and the performance of all aspects of the study, including the methods used for obtaining informed consent, were in accordance with the principles stated in 21 CFR 21.50 (protection of human subjects) and 21 CFR 21.56 (institutional review boards). Isolated PBMC were frozen in RPMI 1640 (Life Technologies, Carlsbad, CA), 20% pooled normal human serum, and 10% DMSO (Sigma-Aldrich, St. Louis, MO) and used for subsequent separations of dendritic cells (DC) and CD8+ and CD4+ T cells. EBV immortalized B lymphoblastoid cells were generated from PBMC by culturing 1 × 106 cells with 5 ml of EBV supernatant and 1 μg/ml cyclosporin A (Novartis, East Hanover, NJ). EBV supernatant was generated from conditioned medium of B95-8 cells (CRL-1612). SCLC cell lines H69 (HTB-119), DMS-79, NCI H126 (HTB-120), HTB-171, and HTB-175; large cell lines HTB-177 and HTB-183; and bronchocarcinoma carcinoma A549-1 were purchased from American Type Culture Collection (Manassas, VA). Adenocarcinoma cell line 180T and HTB, and squamous cell carcinoma cell line 767T and squamous cell carcinoma 767T, 936T, 3,09T, and 114-87T were provided by Dr. J. Siegfried (University of Pittsburgh, Pittsburgh, PA). Adenocarcinoma cell line LA10-1 and squamous cell carcinoma cell line LT-1 were provided by Dr. E. Repasky (Roswell Park Cancer Center, Buffalo, NY), SCLC cell line 659-43; adenocarcinoma cell lines LT 140-98, LPE 86-52, 522-23, and 659-22; and large cell carcinoma cell line 391-06 were generated from primary lung pleural effusion (LPE) or primary lung tumor samples at Corixa. All samples were typed using immunohistochemistry by Phenopath (Seattle, WA). Primary LPE and primary tumor samples were provided by Dr. R. Ostenson (Good Samaritan Cancer Center, Puyallup, WA) and Dr. E. Repasky. Lung tumor cells were transduced with the pBIB retrovirus expressing HLA alleles and were selected two to four times with 20 μg/ml blastidicin-D for 48–72 h. Primary fibroblasts were grown from a skin biopsy of donors in fibroblast-FBS medium (Wathen's medium (Life Technologies, Gaithersburg, MD), 15% FBS (HyClone Laboratories, Logan, UT), 50 μM penicillin (Life Technologies), 50 μg/ml streptomycin (Life Technologies), and 2 μM 1-glutamine (Life Technologies)). Primary fibroblasts were transduced with the pBIZ retrovirus containing B7.1 and were selected twice with 100 μg/ml zeocin for 48 h. B7.1-transduced fibroblasts were then transduced with the retrovirus containing either SOX-4 or irrelevant Ags. The pBIB-transduced cells were selected twice with 20 μg/ml blastidicin-D for 48–72 h. After selections and expansion, transgene expression by fibroblasts was confirmed by FACS analysis using the anti-SOX-4 polyclonal serum described below.

Real-time PCR analysis

Primary lung tumors samples were obtained from Cooperative Human Tissue Network, National Disease Research Interchange, and Roswell Park Cancer Center. Normal tissue samples were obtained from the above sources, and RNA for normal tissues was obtained from Clonetch Laboratories (Palo Alto, CA) and Invitrogen (Carlsbad, CA). Tumor cell lines were obtained from American Type Culture Collection or from in-house sources. Quantitative real-time RT-PCR assays were performed as described previously (11), using 300 nM each of SOX4-specific forward (5’-CTTGACATGATTGCATGATT-3’) and reverse (5’-CCTGTGCACATCATGAGCC-3’) primers. To quantitate the expression level of SOX4 in tumor and normal tissue samples, standard curves were generated using known copy numbers (2–10 × 105) of SOX4 and β-actin cDNA, and SOX4 expression in each cDNA sample was normalized to the internal β-actin levels. The final real-time RT-PCR results are reported as copy number of SOX4 per 1000 copies of β-actin.

Generation of overlapping peptides from the primary amino acid sequence of SOX4

The peptides used in this study were synthesized by Mimotopes (San Diego, CA). Peptides were synthesized on a 1-μmol scale in 96-well plate format. Before use, the peptides were lyophilized from a 1/1 mixture of acetонитрил/water and then solubilized in DMSO at a concentration of 10 mg/ml. A total of 92 20-mer peptides overlapping by 15 aa were synthesized to span the entire coding sequence of SOX-4.

Generation of recombinant SOX4-expressing virus vectors

The SOX-4 open reading frame was amplified from a plasmid containing the SOX-4 cDNA (C. S. Bangur, unpublished observations) using the proofreading thermostable polymerase Pwo (Roche, Indianapolis, IN) and standard PCR-based molecular techniques. The consensus Kozak sequence GCCGCCACC was included immediately 5’ of the initiator ATG to maximize translational initiation. Recombinant adenovirus was generated using standard molecular biology methodologies essentially as described previously (14). Particle concentration was determined by OD, and biological activity was determined by a plaque-forming assay on HEK293 cells. Recombinant retroviruses were generated using the Phoenix-AMPHO packaging system and the vectors pBIB (15), in which the blasticidin-S gene is encoded by the same transcript as the inserted genes of interest.

HLA typing

HLA typing of cell lines was performed at Puget Sound Blood Center (Seattle, WA) using the Micro SSP HLA DNA Typing Tray PCR typing kit (One A. Canoga Park, CA). Individual HLA alleles were cloned from cDNA generated from donor B lymphoblastoid cells using Pwo and standard PCR-based molecular techniques. The consensus Kozak sequence GCCGCCACC was included immediately 5’ of the initiator ATG to maximize translational initiation.

Generation and Ag loading of DC

DC were generated essentially as previously described (16). Briefly, PBMC were isolated by separation on a Percoll (Amer sham Pharmacia Biotech, Piscataway, NJ) density gradient, followed by differential adherence and cultured for 5 days at 37°C in 5% CO2 in DC medium (RPMI 1640, 1% human serum, 0.5 μM 2-mercaptoethanol, and 2 μM 1-glutamine) supplemented with 30 ng/ml IL-4 and 50 ng/ml GM-CSF (Immunex, Seattle, WA). After the 5-day culture, nonadherent and nonadherent DC were harvested by vigorous washing. Harvested cells were <90% DC as assessed by morphology and surface expression of CD13, CD14, MHC class II, and CD80. DC were pulsed with peptide solutions essentially as previously described (17). DC were tested for SOX-4-specific T cell lines by ELISPOT assays. ELISPOT assays were performed as described below.

In vitro stimulation of T cells

CD8+ or CD4+ T cells were enriched from thawed PBMC by negative magnetic bead separation, using anti-CD4 or anti-CD8 and the MACS AQUA kit (Miltenyi Biotec, Auburn, CA). FAG staining for CD8, CD4, and CD3 expression was used to determine the percentage of CD8+ or CD4+ cells within the enriched population.

For CD8+ T cells, in vitro stimulation cultures were established essentially as previously described. After a total of four stimulations, cultures were tested for SOX-4-specific activity using IFN-γ ELISPOT assays. SOX-4-specific T cell lines were cloned using anti-CD3 essentially as previously described.

For CD4+ T cells, in vitro stimulation cultures were established essentially as described previously (17). DC were pulsed with two pools of 42 SOX-4-derived 20-mer peptides overlapping by 15 aa, at a concentration of 250 ng/ml of each peptide. After a total of four stimulation cycles, cultures were tested for SOX-4 specificity. SOX-4 specific lines, cultures were expanded by stimulation on specific peptides at 250 ng/ml of each peptide.

T cell assays

CD8+ T cell activity was assessed by specific IFN-γ release using IFN-γ ELISPOT and 51Cr release cytotoxicity assays. CD4+ T cell activity was measured using IFN-γ ELISA and 3H-proliferation assays. For IFN-γ ELISPOT assays, IFN-γ production by CD8+ T cells was detected essentially according to published protocols, using multiscreen 96-well Isolation plates (MAIPs-45; Millipore, Bedford, MA) coated with 10 μg/ml IFN-γ capture Ab (1D4; Mabtech, Nacka, Sweden). ELISPOT plates were read using an ELISPOT counter (Zeiss, New York, NY).

For 51Cr release assays, targets were labeled overnight in culture medium containing 50 μCi of 51Cr. Labeled targets were plated at 1 × 103 cells/well in 96-well, round-bottom plates and incubated with target cells at

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varying E:T cell ratios for 4 h at 37°C. Culture supernatants were harvested onto Luma Plates (Packard, Meriden, CT), and 51Cr released into supernatants was measured using a TopCount scintillation counter (Packard). Maximal and minimal (background) release were determined from wells that contained labeled target cells treated with 1% Nonidet P-40 or medium alone. The percent specific lysis was determined according to the formula: % specific lysis = ([experimental cpm − minimal cpm]/[maximal cpm − minimal cpm]) × 100.

For IFN-γ ELISA assays, IFN-γ production by CD4+ T cells was detected essentially according to published protocols (17), using enzyme immunoassay/RIA plates (Corning Glass, Corning, NY) coated with 4 μg/ml IFN-γ mAb (18891D; BD PharMingen, San Diego, CA). For the assays, 50 μl of assay culture supernatant (from the 24–48 h point) was used. ELISA plates were read at 450–570 nm using the Benchmark Microplate Reader and Microplate Manager 4.0 (Bio-Rad, Hercules, CA).

For proliferation assays, specific stimulation by CD4+ T cells in response to SOX-4 Ag was measured essentially as previously described (17), using 1 μCi/well [3H]thymidine (Amersham Pharmacia Biotech) added to assay cultures at 48 h. After incubation for 12–16 h, cultures were harvested onto Unifilter plates (Packard). Microscint 20 scintillation fluid (Packard) was added to each well, and plates were counted on the TopCount scintillation counter (Packard).

Cell lysates were generated from SOX4-expressing SCLC cell lines H69 and DM79 and large cell lung carcinoma cell line HTB-183. Cells were resuspended at 106/ml in distilled water, samples were subjected to three or four freeze-thaw cycles, and 0.1 vol of 10× PBS (Life Technologies) was added to the lysates.

Detection of SOX-4-specific Ab responses
Sera from SCLC and healthy donors were evaluated for the presence of anti-SOX-4 Abs using standard ELISAs. Briefly, individual 20-mer peptides overlapping by 15 aa and covering the entire coding sequence of SOX-4 were added to wells of 96-well plates (Nunc, Roskilde, Denmark) at 1 μg/ml in bicarbonate buffer and coated overnight at 4°C. Wells were blocked with PBS/10% nonfat dry milk. After washes, sera, diluted with PBS that contained 10% goat serum initially at 1/300 and then in 3-fold dilutions to 1/72,900, were added to wells and incubated for 1 h at room temperature. Plates were developed with HRP-conjugated rabbit anti-human Ig Abs (Jackson Immunoresearch, West Grove, PA) and substrate (TMS EIA substrate; Bio-Rad). Ab reactivity was determined by a signal-to-noise ratio of >5.

FACS analysis
Cells were stained with a mixture of CD4 FITC, CD8 PE, and CD3 PerCP (BD PharMingen). After washing, cells were analyzed using a FACS Calibur (BD Biosciences, San Diego, CA).

Results
Quantitative real-time PCR analysis of SOX-4 expression in lung tumors
We have recently described the strong overexpression of SOX-4 in primary and established SCLC tumors as well as a pool of primary adenocarcinoma samples (11). To examine in more detail the overexpression of SOX-4 in NSCLC and normal tissues, quantitative real-time PCR analysis was performed using a panel of RNA samples from primary NSCLC, including adenocarcinoma, squamous cell carcinoma, and large cell lung carcinoma; a panel of adenocarcinoma LPE and neuroendocrine carcinoid samples; and a panel of normal essential tissues, using primers derived from the 3′-untranslated sequence of SOX-4. Strong overexpression (>10-fold expression levels in normal lung) of SOX-4 was detected in 7 of 21 adenocarcinoma samples, and overexpression (>3-fold expression levels in normal lung) was found in 7 of 21 adenocarcinoma samples. Overexpression of SOX-4 was observed in one of four LPE samples, and in one of two each of squamous and large cell lung carcinoma samples. Compared with tumor samples, low levels of expression of SOX-4 message (within 3-fold the levels in normal lung) could be detected in a number of normal essential tissues, including two kidney samples, two pancreas samples, and one each adrenal, pituitary, thymus, and thyroid gland samples (Fig. 1).

The expression of SOX-4 was also evaluated in a panel of lung tumor cell lines representing SCLC and NSCLC tumors. As shown in Fig. 2, SOX-4 was strongly overexpressed in four of seven SCLC cell lines and was overexpressed in three of seven SCLC, five of nine adenocarcinoma, two of six squamous cell, one of five large cell, and one of one bronchoalveolar carcinoma cell lines. No overexpression was observed in three aden-squamous carcinoma cell lines.

Identification and characterization of SOX-4-specific CD4+ and CD8+ T cells in PBMC
SOX-4-specific CD4+ T cells. To determine the presence of SOX-4-specific CD4+ T cells in PBMC that could be activated by vaccination, in vitro stimulation experiments were performed. Using PBMC from a healthy donor, stimulation cultures were established in a 96-well format as previously described (17), using as
FIGURE 2. SOX-4 mRNA is overexpressed in established lung tumor cell lines. Expression of SOX-4 mRNA in normal lung, primary lung tumor pools, and a panel of lung tumor cell lines was determined by quantitative real-time PCR analysis using gene-specific primers. The real-time panel was comprised of 33 cDNA samples derived from lane 1 and 2, normal lung; SCLC cell lines: lane 3, NCI H69 (HTB-119); lane 4, DMS-79; lane 5, NCI H128 (HTB-120); lane 6, 659-43; lane 7, HTB-171; lane 8, HTB-175; lane 9, SCLC primary tumor pool, adenocarcinoma cell lines: lane 10, LT 140-98; lane 11, 574-92; lane 12, 86-52; lane 13, 98T; lane 14, LA10-1; lane 15, 522-23; lane 16, 659-22; lane 17, 180T; lane 18, adenocarcinoma primary tumor pool; squamous cell carcinoma cell lines: lane 19, 3-90T; lane 20, TL-1; lane 21, 76T; lane 22, 114-87T; lane 23, 936T; lane 24, squamous cell carcinoma primary tumor pool; large cell carcinoma cell lines: lane 25, HTB-177; lane 26, 81-86T; lane 27, 391-06; lane 28, HTB-183; lane 29, large cell carcinoma primary tumor pool; adeno-squamous carcinoma cell lines: lane 30, 94T; lane 31, 343T; lane 32, 110-87T; bronchoalveolar carcinoma cell line: lane 33, A549-1. The expression of SOX-4 mRNA in each sample was normalized to the internal β-actin levels and is reported as copies per 1000 copies of β-actin. The dotted line represents the 3-fold overexpression cutoff, and the dashed line represents the 10-fold overexpression cutoff compared with average normal lung expression.

stimulator cells DC pulsed with pools of 20-mer peptides overlapping by 15 aa and spanning the entire amino acid sequence of SOX-4. Peptides were combined into two pools, with each pool consisting of 42 sequential peptides. For each peptide pool, 288 independent microcultures (three 96-well plates) were established. After a total of four weekly stimulations, cultures were screened for SOX-4-specific T cell activity using IFN-γ ELISA and 3H incorporation. Seventy lines were identified that specifically recognized autologous monocytes pulsed with SOX-4-derived peptides, as determined by a stimulation index of at least 25 in the 3H incorporation assay and at least 3 in the IFN-γ ELISA. Each of these lines was restimulated on the SOX-4 peptide pool and tested for specific recognition of individual SOX-4-derived peptides. In instances where two distinct nonoverlapping peptide specificities were identified, the line was split and stimulated independently with each peptide specificity. After stimulation with the relevant peptide(s), lines were tested for the ability to specifically recognize autologous DC pulsed with lysates from the tumor cell lines H69 and DMS-79, which express relatively high levels of SOX-4 mRNA, and the large cell carcinoma line HTB-183, which expresses relatively low amounts of SOX-4 mRNA. Two lines, 1A-H8 and 2B-A4, were identified that specifically recognized autologous DC pulsed with lysates from the small cell lung tumor cell lines H69 and DMS-79 that express high levels of SOX-4, but not lysates from the HTB-183 large cell carcinoma cell line that express significantly lower levels of SOX-4. Before stimulation with individual peptides, line 1A-H8 contained T cells that recognized two distinct peptide specificities (Fig. 3A). One specificity was contained in two overlapping peptides (peptides 13 and 14) corresponding to aa 61–85 of SOX-4, and the other specificity was contained in three overlapping peptides (peptides 20–22) that spanned aa 96–125 of SOX-4. Only T cells stimulated on the three overlapping peptides that spanned aa 96–125 of SOX-4 could specifically recognize DC pulsed with SOX-4-expressing tumor cell lysates (data not shown). The core sequence shared by the three peptides corresponds to the sequence IPFIREAERL (aa 106–115 of SOX-4). Line 2B-A4 recognized two overlapping peptides (peptides 91 and 92) that spanned aa 451–474 of SOX-4, with the core sequence shared by the two peptides corresponding to the sequence SEMISGDLWLESLISNL (aa 454–470 of SOX-4; Fig. 3A).

To determine the relative sensitivity of lines 1A-H8 and 2B-A4 to respond to DC pulsed with lysates from lung tumor cells that
expressed SOX-4, each of the lines was stimulated with autologous DC pulsed with titrations of the relevant peptide pools or lysates from the SOX-4-expressing tumor cell lines H69, DMS 79 (that express relatively high levels of SOX-4 message) and HTB-183 (that expresses significantly lower amounts of SOX-4 message), and the ability of lines to respond to the pulsed APC was measured by $^3H$ proliferation assay and IFN-γ ELISA (Fig. 3B). Both lines 1AH8 and 2BA4 recognized APC pulsed with 250 pg/ml peptide, but did not respond significantly to APC pulsed with lower concentrations of the relevant peptide pools. Both lines also specifically and robustly recognized autologous DC pulsed with lysates from tumor cells that expressed relatively high concentrations of SOX-4 message at dilutions of tumor lysates as low as 0.1%. These results demonstrate that each of the CD4 T cell lines recognized SOX-4-derived peptides that were naturally processed and presented in the context of HLA class II molecules on the surface of APC.

To identify the relevant HLA class II allele responsible for presenting the SOX-4-derived epitopes to CD4$^+$ T cells, Ab blocking and HLA mismatch analyses were performed. Ab blocking analysis suggested that the restricting allele was an HLA-DR locus, because anti-HLA-DR, but not anti-HLA-DQ, blocking Abs inhibited recognition of peptide-pulsed APC by each line (data not shown). For the HLA mismatch analysis, a panel of monocytes derived from donors partially HLA matched at class II alleles was pulsed with the relevant peptides and used as APC for each of the T cell lines in IFN-γ ELISA and $^3H$ proliferation assays. APC derived from the autologous donor 331 and donors 216, 206, 385, 77, 138, and 142 could stimulate each of the SOX-4-specific CD4$^+$ T cell lines, whereas APC from donors 363, 12, and 326 failed to stimulate the SOX-4-specific T cells (Fig. 3C). As the only HLA-DR allele expressed by the stimulating APC, but not the nonstimulating APC, was the HLA-DRB*01 allele, these data demonstrate that for both lines 1A-H8 and 2B-A4, the restricting allele for the SOX-4-specific CD4$^+$ T cell response is the HLA-DRB*01 allele.

SOX-4 is a member of a multigene family that includes at least 14 family members (18). SOX-4 is a member of the C subgroup of SOX proteins that includes SOX-11 and SOX-22. To examine the homology of the CD4 epitopes identified in this work between SOX-4 family members, a comparative amino acid alignment was performed. The results of this analysis are presented in Fig. 4. SOX-4-specific T cell clones were identified from two lines, 5E-12 and 3C-7. Representative clones from each line were further expanded and characterized. On the basis of HLA restriction and SOX-4 truncation analyses, clones from each line appeared to have the same SOX-4 specificity; therefore, data are presented for one clone, 5-E12-E9.

The HLA class I allele responsible for the presentation of the SOX-4-derived peptides to 5-E12-E9 was identified by Ab blocking and HLA mismatch IFN-γ ELISPOT assays. Ab blocking assays indicated that the restricting allele was an HLA-B* or -Cw* allele, because the ability of the clone to recognize autologous fibroblasts transduced to express SOX-4 was blocked by both anti-class-I and anti-HLA-C Abs, but not by anti-HLA-A2 or control Abs (data not shown). For the HLA mismatch analysis, a panel of fibroblasts HLA matched with the donor at the individual HLA-B and -Cw alleles was transduced to express SOX-4 or an irrelevant Ag, and the ability of 5-E12-E9 to recognize transduced fibroblasts was examined in IFN-γ ELISPOT assays. Only fibroblasts that were transduced to express SOX-4 and also expressed the HLA-Cw*1402 allele were recognized by 5-E12-E9 (data not shown). These results indicated that the restricting allele for the SOX-4-specific response is the HLA-Cw*1402 allele. To identify the minimal SOX-4-derived epitope recognized by the clone, truncation analyses were pursued. These analyses indicated that the epitope recognized by the T cells was localized to a subfragment of SOX-4 that was contained in part within aa 91 and 284 of SOX-4. Although the truncation analysis identified a subfragment of SOX-4 that contained the relevant epitope, extensive efforts using overlapping 9- and 10-mer peptides derived from the

**FIGURE 4.** Homology alignment of SOX-4 CD4$^+$ T cell epitopes with SOX-11 and SOX-22. Protein sequence alignments of the 1A-H8 peptide epitope SOX-4$^{906-115}$ and the 2B-A4 peptide epitope SOX-4$^{455-470}$ with known members of the SOX gene family of transcription factors.
subfragment failed to identify the minimal peptide. These results suggest that the minimal peptide epitope recognized by clone 5-E12-E9 may be different from a 9- or 10-mer, may be subject to modification, or, alternatively, may be encoded from alternative reading frames of SOX-4.

To determine whether the epitope recognized by the 5-E12-E9 clone was naturally processed by lung tumors, the SOX-4-expressing squamous lung tumor cell line 390-T, the bronchoalveolar carcinoma line A549-1, and the large cell lung tumor cell line HTB183 that expresses lower, but still detectable, levels of SOX-4 were transduced and selected to express either the HLA-Cw*1402 allele or the irrelevant HLA-B*4402 or -A*0301 allele. SCLC tumor cell lines were not used in these analyses because they were not amenable to stable retroviral transduction. Stably transfected tumor cell lines were then used in IFN-γ ELISPOT assays and standard 51Cr release assays as APC and targets for the 5-E12-A12 clone. As shown in Fig. 5A, in IFN-γ ELISPOT assays the SOX-4-specific T cell clone specifically recognized the SOX-4-positive tumor cell lines transduced with the relevant HLA-Cw*1402 restricting allele, but not the same cell lines transduced with the irrelevant HLA-B*4402 or -A*0301 restricting alleles. Although 390-T produces significantly higher levels of SOX-4 transcript than HTB-183 and A549-1, the clone produced similar levels of IFN-γ in response to both cell lines. As shown in Fig. 5B, in 4-h 51Cr release assays, the 5-E12-E9 clone specifically recognized and lysed the 390-T cell line transduced with the relevant HLA Cw*1402 allele, but not the same cells transduced with the irrelevant HLA-B*4402 allele. The 5-E12-E9 clone also specifically recognized HTB-183 cells transduced with the relevant HLA Cw*1402 allele, but not the irrelevant HLA-B*4402 allele; in agreement with the lower levels of SOX-4 expression by HTB-183, the 5-E12-E9 clone demonstrated significantly lower cytosis of the HTB-183 targets. No specific lysis of the A549-1 cell line could be detected, suggesting that these cells may be resistant to perforin/granzyme-mediated cytosis (data not shown).

**Detection of SOX-4 specific Abs in sera from lung cancer patients**

The presence in cancer patients of an Ab response specific for a tumor Ag is evidence for the development of a humoral and presumably an accompanying CD8+ T cell immune response. To determine whether SOX-4 was immunogenic in that context, we assessed the presence of SOX-4-specific IgG Abs in sera from SCLC and normal healthy individuals. We were unable to evaluate the presence of SOX-4-specific Ab responses in NSCLC patients because those materials were not available to us. Using ELISAs, we examined sera from 25 SCLC patients and 23 healthy donors for the presence of SOX-4-specific Abs that could recognize each of 92 linear overlapping 20-mer epitopes spanning the entire sequence of SOX-4. SOX-4-specific Ab responses were observed in nine of 25 SCLC patients. These Abs recognized eight independent SOX-4-derived epitopes (Table I). Western analyses using recombinant *Escherichia coli*-generated SOX-4 and an equivalently prepared irrelevant protein demonstrated that the SOX-4-reactive sera were able to specifically recognize purified, *E. coli*-generated recombinant SOX-4 (data not shown). No Ab responses could be detected in 23 healthy donors. SOX-4-specific Ab titers were detected at dilutions of 1/10^3 to 1/10^5, titers that compare favorably with titers reported in patients for other immunogenic tumor Ags, such as NY-ESO-1 (19). Four of the peptides, corresponding to AGESSDAGGAEGLIASSPT (aa 126–145), YKYRPRKKKVKSG NANSSSSA (aa 126–145), KHLAEKKVKRVLFGGLGTS (aa 286–305), and PLGLYEEEGACSPDAPSL (aa 321–340) were identified as common epitopes recognized by sera from multiple SCLC patients.

**Table I. Prevalence and epitopes of SOX-4-specific Abs in SCLC patients**

<table>
<thead>
<tr>
<th>SOX-4 Peptide</th>
<th>Amino Acid Span</th>
<th>SCLC Patients</th>
<th>Healthy Donors</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>6–25</td>
<td>1</td>
<td>0</td>
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<td>48</td>
<td>236–255</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>58</td>
<td>286–305</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>65</td>
<td>321–340</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Total positive 9/25 0/23

% Positive 36 0

* Sera were tested by ELISA for specificity against individual 20-mer peptides overlapping by 15 aa and spanning the entire sequence of SOX-4. The table only contains data for peptides that demonstrated reactivity with sera; all other peptides were nonreactive. Ab reactivity was determined by a signal-to-noise ration of >5. Sera from a total of nine patients demonstrated reactivity to one or two individual peptides.
SCLC patients. Sequence analysis revealed that each of these common epitopes was SOX-4 specific, with no significant homology to any other protein in the GenBank protein database (data not shown). Although a correlation of Ab positivity and SOX-4 expression by tumors would have been optimal, we were unable to perform such an analysis because patient tumor material was not available for us to evaluate. Additionally, because the amount of patient blood that we were able to obtain was extremely limited, we were not able to evaluate the presence of a SOX-4-specific T cell repertoire in the patients who demonstrated SOX-4 Ab responses.

Discussion
A considerable amount of effort has been applied over the past decade toward the discovery and development of candidate molecules for use in vaccine-based immunotherapy strategies against cancer. Experience from both experimental models and clinical trials has revealed a number of characteristics that are considered desirable for vaccine candidates. To allow for broad use, a prerequisite for vaccine candidates is that the molecule be expressed in a significant fraction of the target tumor type(s). Additionally, because of the potential for autoimmunity, one important characteristic of molecules under consideration for vaccines is that they be expressed in a tumor-specific manner or, at a minimum, overexpressed in tumor vs normal cells. Finally, due to the potential for Ag loss tumor cell variants after anti-tumor immunotherapy (20), ideal vaccine candidate molecules should also be involved in the tumor phenotype.

A critical requirement for ultimate efficacy of vaccines is the presence of a precursor Ag-specific T cell repertoire that be activated in response to vaccination. Although Ag-specific CD8\(^+\) T cells have historically been thought to be the critical effector cells important for an effective antitumor response, it is becoming increasingly evident that Ag-specific CD4\(^+\) T cells also play an important role in this process. In particular, CD4\(^+\) T cells have been shown to play a critical role in tumor eradication when CD8\(^+\) T cell numbers are suboptimal (21), to be involved in both CD8\(^+\) T cell maintenance and tumor mass infiltration (22), to be important for the recruitment of antitumor effector cells to the tumor mass (23), as well as to have direct antitumor effector functions (24). CD4\(^+\) T cells have also been shown to be important for the maturation of DC, a process critical for the effective activation of the Ag-specific CD8\(^+\) T cell compartment (25). Finally, the activation of tumor Ag-specific CD4\(^+\) T cell help is an important prerequisite for the subsequent development of a humoral antitumor response.

The existence of a comprehensive immune response specific for vaccine candidate Ags has been demonstrated in very few cases, most notably for MAGE-A1, MAGE-A3, NY-ESO-1, and Her-2/neu, and each of these Ags is currently in clinical trials as tumor Ag vaccine candidates.

In this report we evaluate the potential of the transcription factor, SOX-4, as a vaccine candidate for lung cancer. We demonstrate that on the basis of expression levels, coverage of lung tumors, and SOX-4-specific immune repertoire, SOX-4 is an excellent candidate for development as a lung cancer vaccine. Specifically, SOX-4 mRNA is shown to be significantly overexpressed in a number of primary and established lung tumor types, including SCLC and NSCLC (adenocarcinomas, squamous, and large cell lung carcinoma) specimens. Furthermore, using in vitro stimulation protocols, we demonstrate in PBMC from healthy donors the presence of SOX-4-specific CD8\(^+\) and CD4\(^+\) T cell precursors that recognize naturally processed epitopes from SOX-4. Finally, we demonstrate the presence of SOX-4-specific Abs in sera from lung cancer patients.

SOX-4 mRNA was strongly overexpressed (>10-fold) in 33% of primary adenocarcinoma and was overexpressed (>3-fold) in an additional 33% of adenocarcinoma samples compared with levels in normal lung. Although there are a number of gene products that are overexpressed by tumors that are being evaluated as vaccine candidates (most notably the melanoma differentiation Ags), quantitative measurements for the degree of overexpression in tumor vs normal tissues are for the most part lacking. Nonetheless, the fold overexpression of SOX-4 in tumor samples compared with normal lung compares favorably with other tumor overexpressed Ags, such as MUC-1, p53, and mammaglobin (26, 27).

There is ample precedence in the literature for presentation of epitopes derived from an intracellular Ag, such as SOX-4 to CD8 and CD4 T cells, and at least some of the mechanisms responsible have been elucidated (28). In the case of class I-restricted epitopes, direct processing of intracellular SOX-4 by the proteasomal machinery of the tumor cells would generate the relevant epitopes assembled with class I and ultimately exported to the cell surface to be recognized by T cells. In the case of class II-restricted T cell epitopes, the most likely source of SOX-4 protein is material released from dying cells within the tumor mass; released SOX-4 protein would be taken up by APC, processed, assembled with class II, and ultimately presented on the cell surface to stimulate T cells.

SOX-4 is a member of the SOX protein family, which has at least 14 family members (18). SOX proteins function as transcription factors and modulators of chromatin architecture and are involved in various aspects of embryonic development, such as sex determination, neural development, lens development, chordogenesis, and hemopoiesis (18). All SOX proteins contain a DNA-binding domain that is highly similar to the HMG box domain, and based on the sequence conservation within the HMG box, SOX family members are subdivided into seven subgroups. SOX-4 belongs to the C subgroup, which also includes SOX-11 and SOX-22. SOX-4 has been shown to be involved in endocardial ridge and B cell development, and is expressed in a number of tissues during embryonic development, including embryonic heart, the developing CNS, lung, tooth buds, gonads, mesonephros, as well as the thymus and pre-B and T cells (18). In mice, SOX-4 has been shown to facilitate thymocyte differentiation (29).

Although a number of SOX family members have been found to be associated with cancer, the role of SOX proteins in tumorigenesis remains unclear. Like other HMG box proteins (30, 31), some SOX family members may be directly involved in the malignant transformation and development of the cancer phenotype. Ectopic expression of SOX-3 in chicken embryo fibroblasts causes oncogenic transformation (32). Evidence has been presented suggesting that SOX-4 also may be involved in tumorigenesis. In particular, SOX-4 was found to be one of the most frequently targeted common retroviral integration sites in retroviral insertional mutagenesis studies designed to identify genes involved in tumorigenesis (12, 13). In addition to lung cancer, both SOX4 and SOX11 have been shown to be differentially expressed in medulloblastoma and other neuronal malignancies (33) (C. Bangur, unpublished observations). Other members of the SOX family are also observed to be expressed in cancer tissues, including lung, brain, pancreas, stomach, and breast cancers (11, 34, 35).

The observation that lung tumors commonly lose class I expression (36) suggests that CD8\(^+\) T cells are involved in the control of lung cancer at some stage of tumor progression. Thus, the presence of a SOX-4-specific CD8\(^+\) T cell repertoire provides support for the development of SOX-4 as a vaccine. The data presented in this report demonstrate that a CD8\(^+\) T cell repertoire exists in humans.
that is specific for SOX-4 and can therefore be targeted for activation by the appropriate vaccination strategies. The CD8+ T cells described in this work expressed significant, but moderate, effector functions, as assessed by both IFN-γ production and target cell cytolysis, and thus it is reasonable to speculate that successful activation of these cells would have an antitumor effect against tumor cells that express class I. Finally, the CD8+ T cells identified in this work are restricted by the HLA-Cw*14 allele that is expressed in a low frequency of individuals (roughly 2–8% of individuals in the U.S.) (37). Nonetheless, it is reasonable to suspect that additional SOX-4–specific CD8+ T cells, restricted by more common HLA class I alleles and with similar effector phenotypes to the T cells described in this report, exist in PBMC of lung cancer patients.

The frequent loss of class I expression by lung tumors imparts particular significance to the presence of a SOX-4–specific CD4+ T cell compartment that could be activated by appropriate vaccine strategies against lung cancer. The ability to activate and maintain Ag-specific CD4+ T cells at the tumor site may be critical for the production of cytokines leading to the recruitment of effector cells at the tumor site as well as for the development of an antitumor Ab response.

Evidence has been presented that demonstrates a role for CD4+ T cells in lung cancer. Specifically, an evaluation of the immunological factors that were associated with a favorable prognosis in lung cancer demonstrated a statistically significant relationship between HLA-DR expression and survival for both squamous and small cell carcinoma (38). In the same study, poor prognosis was correlated with decreased CD4+/CD8+ T cell ratios. CD4+ T cells present in the PBMC of a lung cancer patient have been shown to suppress the growth of an autologous lung tumor in an SCID mouse model (39). Finally, it has been recently demonstrated that through interacting with eosinophils, Th2 CD4+ T cells, were able effectively clear CTL-resistant lung tumor metastases in a mouse melanoma model (40). The demonstration and characterization of SOX-4–specific CD4+ T cells in PBMC provide the first detailed description of the CD4+ T cell repertoire to a lung tumor vaccine candidate. These CD4+ T cells demonstrated significant cytokine activity and robust proliferation in response to lysates from tumor cells that expressed SOX-4. The epitopes recognized by each of the CD4+ T cell lines demonstrated considerable homology with SOX-11 and SOX-22, the other two members of the SOX-4 subfamily. SOX-11 in particular has been shown to be significantly overexpressed by SCLC as well as neuronal tumors with low levels of expression in normal neuronal tissues (C. Bangur, unpublished observations). Thus, a SOX-4 vaccine could potentially show efficacy against tumors that express SOX-11 and/or SOX-22. Because strong humoral immune responses cannot be detected against most tumor Ags, the relationship between the development of Ab responses and clinical outcome has not been rigorously evaluated. However, the cancer/testis Ag NY-ESO-1 provides one system to evaluate the contribution of the humoral response to antitumor immunity. NY-ESO-1 is considered to be a very immunogenic Ag, with strong CD4 and CD8+ T cell as well as Ab responses detected in patients (41). The presence of Ab responses to NY-ESO-1 has been correlated with a positive clinical outcome (42). The SOX-4–specific Ab responses described in this work compare very favorably in both frequency and magnitude with the responses described for NY-ESO-1 and provide further support for the conclusion that a SOX-4 vaccine may be capable of eliciting a comprehensive antitumor immune response.

Although clear overexpression of SOX-4 was observed in lung tumor samples, lower levels of SOX-4 message could also be detected in a number of normal essential tissues, including normal lung, kidney, pancreas, as well as adrenal, pituitary, thymus, and thyroid glands. Preliminary immunohistochemical analysis using polyclonal anti-SOX-4 sera supports the mRNA analysis, with apparent specific staining of pancreatic islet cells and weak staining of a number of tissues, including bronchial epithelium. The low level expression of SOX-4 in normal tissues suggests that the endogenous SOX-4–specific CD4+ and CD8+ repertoire has probably been shaped by peripheral tolerance. The low level expression of SOX-4 in normal tissues also raises the possibility that successful activation of SOX-4–specific T cells may lead to autoimmunity. Thus, evaluation of the potential to elicit autoimmunity by vaccination with SOX-4 will be a critical issue to address before the clinical development of SOX-4. A substantial body of literature exists demonstrating that induction of T cell and Ab responses to tissue-specific Ags that are overexpressed by tumors can lead to antitumor effects without compromising autoimmunity (43–45). However, evidence also exists in the literature that severe autoimmunity can result when tissue-specific Ags are used in DC based immunotherapy (46). A recent study using transgenic mice that expressed the immunodominant FmuLV gag epitope in the liver demonstrated that CD8+ T cells specific for this Ag that were tolerized could be recovered, but could be rendered functionally competent after in vitro stimulations and, once activated, could eradicate tumor in vivo without accompanying liver autoimmunity (47). The development of effective whole gene-, protein-, or peptide-based vaccine strategies that include appropriate adjuvants and delivery systems to effectively activate the SOX-4–specific T cell repertoire in vivo to eradicate tumors without autoimmunity is an important objective to allow the use of this Ag as a lung cancer vaccine.

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References

suppression subtractive hybridization and cDNA microarray expression analysis. 

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