

# Characterization of Mouse Spleen Cells by Subtractive Proteomics\*<sup>§</sup>

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**Major analytical challenges encountered by shotgun proteome analysis include both the diversity and dynamic range of protein expression. Often new instrumentation can provide breakthroughs in areas where other analytical improvements have not been successful. In the current study, we utilized new instrumentation (LTQ FT) to characterize complex protein samples by shotgun proteomics. Proteomic analyses were performed on murine spleen tissue separated by magnetic beads into distinct CD45<sup>-</sup> and CD45<sup>+</sup> cell populations. Using shotgun protein analysis we identified ~2,000 proteins per cell group by over 12,000 peptides with mass deviations of less than 4.5 ppm. Datasets obtained by LTQ FT analysis provided a significant increase in the number of proteins identified and greater confidence in those identifications and improved reproducibility in replicate analyses. Because CD45<sup>-</sup> and not CD45<sup>+</sup> cells are able to regenerate functional pancreatic islet cells in a mouse model of type I diabetes, protein expression was further compared by a subtractive proteomic approach in search of an exclusive protein expression profile in CD45<sup>-</sup> cells. Characterization of the proteins exclusively identified in CD45<sup>-</sup> cells was performed using gene ontology terms via the Javascrypt GoMiner. The CD45<sup>-</sup> cell subset readily revealed proteins involved in development, suggesting the persistence of a fetal stem cell in an adult animal. *Molecular & Cellular Proteomics* 4:1459–1470, 2005.**

The use of proteomic technologies for global characterization of proteins expressed in cells, tissues, and biological fluids is a key component in furthering our ability to understand biological processes in normal and diseased states. In many proteomic studies, cells or tissues are characterized by profiling and comparing proteins expressed in treated and untreated cells, cancerous *versus* normal tissues, or various cell populations. A seminal tool in this development has been the application of mass spectrometry technologies to identify proteins and post-translation modifications in complex protein mixtures. The term “shotgun proteomics” is used to de-

scribe mass spectrometry-based methods that enable the rapid identification of proteolytic peptides from complex protein mixtures in a data-dependent manner (1). Two major analytical challenges encountered by shotgun proteome analysis include the significant variability of protein abundance (dynamic expression range) and the diversity of protein expression (multiple protein forms) in protein mixtures (2), which combine to produce many hundreds of thousands of individual peptide species in the final analysis, often spanning 6 orders of magnitude in abundance. Such issues are only partially addressed with the simple expansion of chromatographic peak capacities in multidimensional separations (3, 4).

Due in part to the dynamic range challenge, recent proteomic studies have profiled proteins expressed in cellular organelles rather than whole cell extracts. By reducing initial mixture complexity, investigators have been able to identify lower abundance proteins involved in nuclear pore trafficking (5), centrosome function (6), and chromatin organization (7, 8) increasing our knowledge of basic cellular physiology. Because mass spectrometers are generally poor at measuring quantitative differences in peptide concentration purely by ion intensity, a number of methods have been developed to measure biological changes between samples. These include a diverse set of labeling methods including stable isotope labeling with amino acids in cell culture (SILAC)<sup>1</sup> (9) and the ICAT (10) strategies. In addition, investigators have also proposed non-stable isotope-based methods to profile protein expression differences between complex mixtures including several based on peptide counting or protein coverage (11–13). In general, relative extent of protein coverage by detected peptides scales with the expression level of the observed protein.

Recently investigators have utilized subtractive proteomics to identify proteins from cellular organelles by subtracting out common protein contaminants. In one elegant example, Schirmer *et al.* (14) utilized a subtractive proteomic technique to identify nuclear envelope proteins with possible disease links. Thirteen known and 67 potentially new integral nuclear membrane proteins were described by removing common nuclear membrane preparation contaminants. Another study

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Received, May 12, 2005, and in revised form, July 14, 2005

Published, MCP Papers in Press, July 21, 2005, DOI 10.1074/mcp.M500137-MCP200

<sup>1</sup> The abbreviations used are: SILAC, stable isotope labeling with amino acids in cell culture; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; AGC, automatic gain control; FP, false positive.

identified candidate substrates for sumoylation in yeast via large scale subtraction of proteomic datasets (15). Subtractive proteomics relies on the exclusive or differential detection of peptides from a given protein during a shotgun proteomic experiment. As might be expected, the effectiveness of subtractive proteomics is greatly influenced by the sampling rate of peptides in the complex mixture and the number of unique peptides identified for each protein. Characterization of the proteome of interest by subtractive proteomics depends primarily on obtaining a pure (approaching 100%) protein extract of at least one of the two samples to be compared.

In the present studies, we performed subtractive proteomic analysis with a new (LTQ FT) and more traditional (LCQ Deca XP) instrumentation to characterize two populations of cells from mouse spleen. The LTQ FT is a novel hybrid mass spectrometer consisting of a linear ion trap and an FT ICR mass analyzer. Conventional three-dimensional (spherical trap) ion trap mass spectrometers exhibit lower mass accuracy and limited ion trapping efficiency. In contrast, the linear (two-dimensional) ion trap of the LTQ has a relatively high ion capacity, a feature that directly results in improved dynamic range. In addition, improvements to the ion source and transfer optics have allowed for increased sensitivity. Huge increases in both resolution and mass accuracy of precursor ions are achieved via mass analysis in the ICR cell (16, 17). With the improved mass accuracy provided by the ICR cell and the substantial increase in scan rate for the LTQ ion trap, this hybrid instrument has the potential to significantly improve shotgun analysis of very complex protein samples.

Protein mixtures were generated by whole cell fractions prepared from CD45<sup>-</sup> and CD45<sup>+</sup> spleen cells. These cell populations were chosen due to the observation that CD45<sup>-</sup> cells were able to regenerate functional pancreatic islet cells in a non-obese mouse model of type I diabetes (18). Because the CD45<sup>-</sup> spleen cell population also contains the stem cells responsible for the regeneration, the stated goal of these experiments was to identify proteins expressed exclusively in CD45<sup>-</sup> cells through subtractive proteomics of the CD45<sup>+</sup> cell fraction. We define subtractive proteomics as the set of proteins identified exclusively by at least two unique peptides in each cell population. When replicates are considered an exclusively identified protein should be identified by at least two unique peptides in any of the replicate studies but not in any of the replicate control samples. The assumption is that the exclusive expression of a protein in one cell population would result in the exclusive detection of peptides from that protein.

After LC-MS/MS analysis using identical chromatographic conditions on both instruments, proteins were identified by database searching using the SEQUEST algorithm (19). Proteins from CD45<sup>-</sup> and CD45<sup>+</sup> cells were compared by a subtractive proteomic approach in search of exclusive expression in CD45<sup>-</sup> cells. To our knowledge no studies have utilized this subtractive proteomic approach to identify differ-

ences between complex tissue samples. Our goal was to evaluate proteins involved in the regeneration of pancreatic islet cells by subtractive proteomics with new and exciting instrumentation.

### EXPERIMENTAL PROCEDURES

*Mice*—Male C57B1/6 mice from The Jackson Laboratory (Bar Harbor, Maine) were maintained under pathogen-free conditions until harvesting of splenocytes at 6 weeks of age for MS analysis.

*Isolation of CD45<sup>-</sup> and CD45<sup>+</sup> Cell Populations from C57B1/6 Mice*—Splenocytes from seven mice were harvested from spleen tissue mechanically disrupted with forceps. Following the lysis of red blood cells (140 mM ammonium chloride in 100 mM Tris buffer, pH 7.5) CD45<sup>+</sup> and CD45<sup>-</sup> spleen cells were separated using mouse-specific CD45 MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly pooled splenocytes were counted, and 10<sup>7</sup> cells were resuspended in 10  $\mu$ l of CD45 MicroBeads in 90  $\mu$ l of 1 $\times$  PBS, pH 7.2 and incubated for 15 min at 4  $^{\circ}$ C. Cells were washed with 1 $\times$  PBS, centrifuged at 1,800 rpm for 5 min, and diluted in 3 ml of 1 $\times$  PBS. Labeled cells were placed in a SuperMACS column in the magnetic field and washed three times with 3 ml of 1 $\times$  PBS to recover the unlabeled, negatively selected CD45<sup>-</sup> cells. The column was removed from the magnetic field, and the positively selected CD45<sup>+</sup> cell fraction was collected in 5 ml of 1 $\times$  PBS.

*Protein Preparation, Separation, and In-gel Digestion*—Whole cell fractions were prepared from either pooled CD45<sup>-</sup> or CD45<sup>+</sup> cell populations using RIPA buffer (1 $\times$  phosphate-buffered saline (Invitrogen), 0.5% deoxycholic acid, 1% Nonidet P-40, 0.1% SDS, 0.02 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 20 mM sodium orthovanadate (Sigma)) and stored at -80  $^{\circ}$ C. Protein concentrations were determined by the Bio-Rad DC protein assay (Bio-Rad), and a total of 1 mg of protein was resolved by one-dimensional polyacrylamide gel electrophoresis using a 4–12% bis-Tris gel on a Novex Mini-Cell (Invitrogen). Proteins prepared from CD45<sup>-</sup> and CD45<sup>+</sup> cells were separated by molecular weight to ~5 cm from the origin in independent 1-well Invitrogen gels using 1 $\times$  MES, SDS running buffer. Gels were removed from the cassette, stained with 0.1% Coomassie Brilliant Blue R250 (Pierce) for 2 min, and destained overnight in a solution of 10% acetic acid and 30% methanol. Gel bands were then excised and used to prepare 14 independent in-gel trypsin (Promega, Madison, WI) digests for each gel as described previously (20). Peptides were extracted by washing gel pieces two times for 20 min at room temperature with a solution containing 5% formic acid and 50% acetonitrile, then dried to complete dryness by vacuum concentration, and stored at -20  $^{\circ}$ C until analysis by mass spectrometry.

*Peptide Sample Preparation*—Prior to analysis, peptide samples were prepared by using in-house nanocolumns to remove excess salt and particulates. Nanocolumns were constructed using Eppendorf Geloader<sup>TM</sup> tips (Brinkmann Instruments) pinched 1 mm from the end and filled to 1.5 cm with Oligo R3 reverse phase packing resin (PerSeptive Biosystems, Framingham, MA) in 100% 2-propanol with the aid of a 1-ml syringe with a flow of 1  $\mu$ l/min without drying the resin. The column was washed with 20  $\mu$ l of 2-propanol and 40  $\mu$ l of elution buffer (97.4% acetonitrile, 2.5% H<sub>2</sub>O, and 0.1% formic acid) and conditioned with loading buffer (98% H<sub>2</sub>O, 0.1% TFA, and 2% acetonitrile). Peptide samples were diluted in 30  $\mu$ l of loading buffer, incubated for 10 min at room temperature, and loaded onto the column. The column was washed with 40  $\mu$ l of 0.1% TFA, and peptides were eluted with 20–30  $\mu$ l of elution buffer. Samples were vacuum-dried and reconstituted in 100–200  $\mu$ l of sample buffer A (95% H<sub>2</sub>O, 5% acetonitrile, and 0.5% formic acid), and 2% was loaded via autosampler for MS analysis.

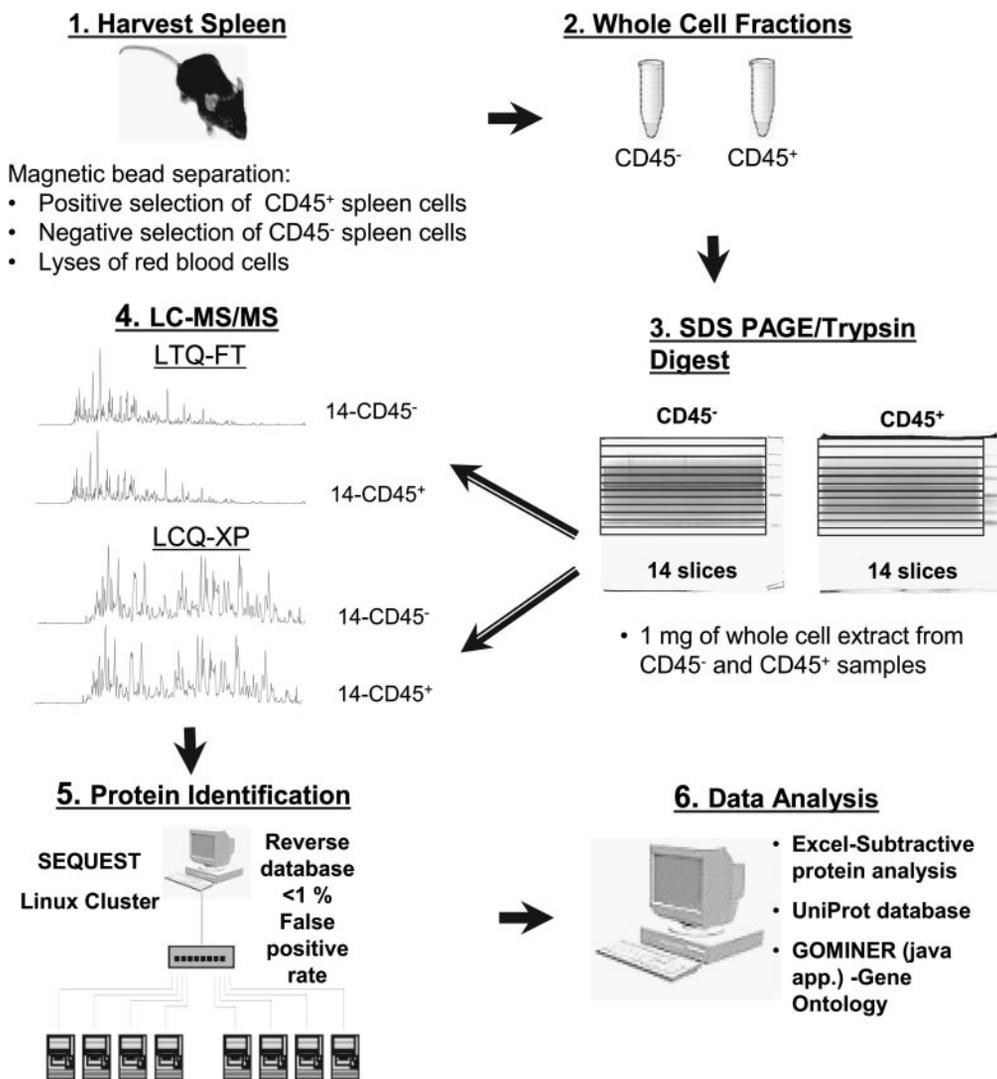


FIG. 1. **Schematic representation of methods.** Splens were removed from C57B1/6 mice and divided into CD45<sup>+</sup> and CD45<sup>-</sup> populations by CD45 microbead separation as described under “Experimental Procedures.” Whole cell protein fractions were prepared and resolved by SDS-PAGE. Fourteen regions were subjected to in-gel digestion with trypsin digests, and identical aliquots were analyzed by LC-MS/MS techniques on both LTQ FT and LCQ XP mass spectrometers. The goal was to compare the utility of new and existing mass spectrometry instrumentation to identify proteins expressed in two spleen cell populations. Proteins were identified using the SEQUEST algorithm (19) on a Linux cluster. Proteins identified by LC-MS/MS were characterized by subtractive protein analysis of the CD45<sup>-</sup> and CD45<sup>+</sup> cell populations, by UniProt and other publicly available databases, and by gene ontology assignment using the GoMiner application.

**LCQ Deca-XP Mass Spectrometry**—LC-MS/MS was performed using an LCQ Deca XP<sup>Plus</sup> ion trap mass spectrometer (ThermoElectron, San Jose, CA). Samples were autoloading (Famos autosampler, LC Packings, Sunnyvale, CA) to a 125- $\mu$ m-inner diameter fused silica C<sub>18</sub> capillary column packed to 14 cm with Magic (Michrom BioResources) C<sub>18</sub> resin (200- $\text{Å}$  pore size, 5- $\mu$ m diameter) using an Agilent 1100 series binary pump with an in-line flow splitter. Peptides were loaded onto the column for 15 min at 120 bars in buffer A (2.5% acetonitrile and 0.15% formic acid). Peptides were then resolved by applying a gradient of 5–33% buffer B (97.5% ACN and 0.15% formic acid) for 55 min at 60 bars. Five MS/MS spectra were acquired per cycle in a data-dependent manner (2).

**LTQ FT Mass Spectrometry**—LC-MS/MS was performed using an LTQ FT hybrid linear ion trap FT ICR MS system (ThermoElectron, San Jose, CA) in similar fashion to the XP with slight modifications. All aspects of the microcapillary separation were identical including the

column, autosampler, sample amounts, HPLC pumps, and HPLC gradient formation. Within the LTQ, 10 MS/MS spectra were acquired per cycle in a data-dependent manner from a preceding FT scan (400–1,800  $m/z$  at a resolution setting of  $10^5$ ) with an automatic gain control (AGC) setting of  $2 \times 10^6$ . Charge state screening was used such that singly charged peptides were not selected, and a threshold of 500 counts was required to trigger MS/MS spectra. Where possible, the instrument operated in a parallel processing mode where the LTQ and ICR cell were both detecting ions.

**Database Searching**—Raw MS/MS data were searched against the mouse NCBI non-redundant database with no enzyme constraint using SEQUEST (19) (version 27, revision 9). Parameters included a precursor mass tolerance of 1.08 and 2.0 Da for LTQ FT and XP data, respectively. Fragment ion tolerance was set at the default, and dynamic modification to methionine (+15.9949) was allowed. Cysteines were searched with a static modification (+71.0370). Only fully

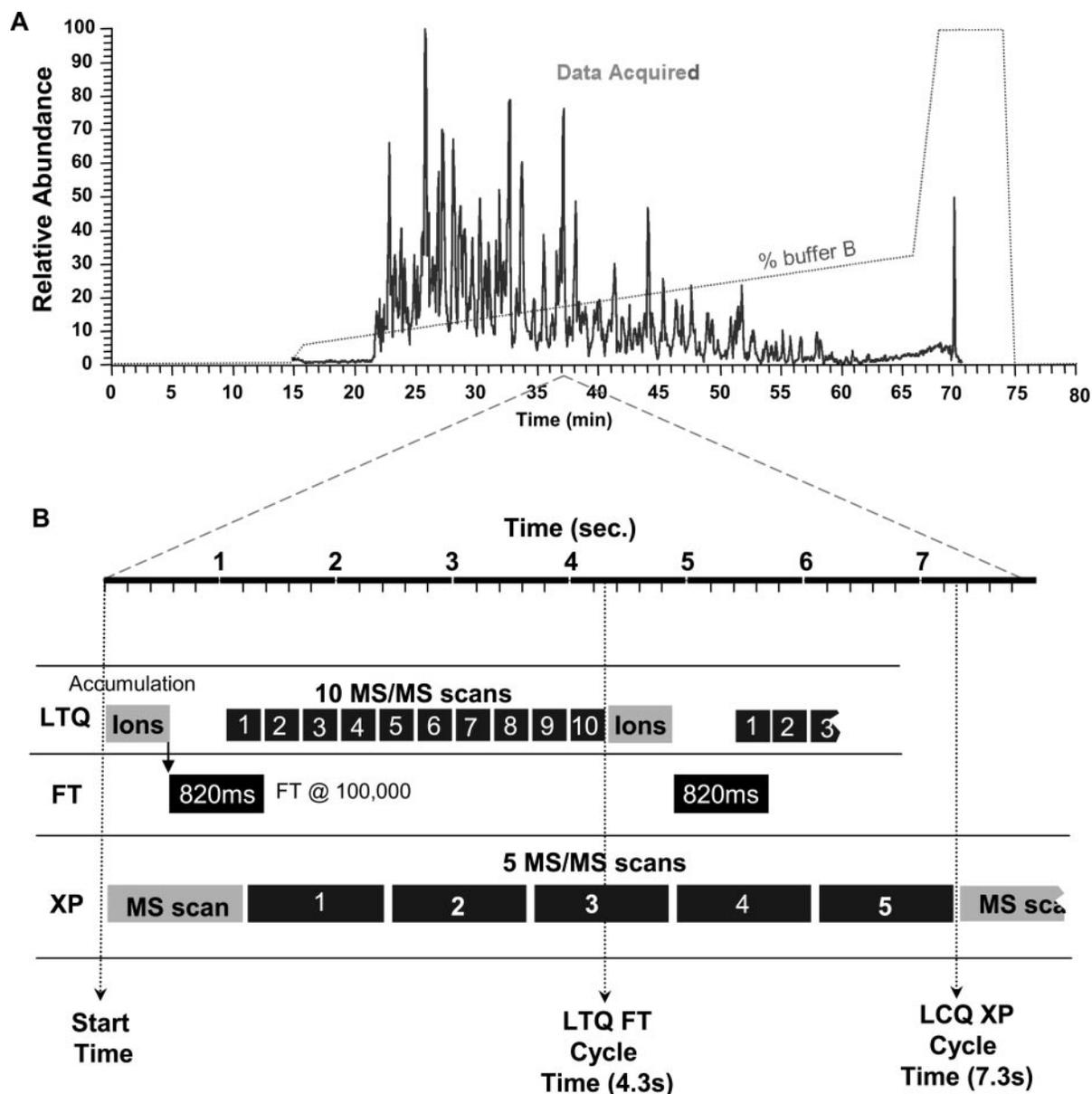


FIG. 2. **Example of LC-MS/MS analysis of one gel region.** A, base peak chromatogram showing one of 14 CD45<sup>-</sup> gel regions analyzed by the LTQ FT. Identical samples were analyzed by LTQ FT and LCQ XP for 80 min with data acquired for 55 min as shown. The gradient used for all experiments is indicated by the percentage of solvent B along the 80-min analysis. B, schematic representation of the standard workflow of the LTQ FT and LCQ XP. The increased scan rate of the LTQ FT enabled a significant increase in the number of peptide ions selected for MS/MS analysis and in the number of peptides and proteins identified over an average cycle time of 4.3 s for the LTQ FT and 7.3 s for the LCQ XP.

tryptic peptides were considered for further processing with Xcorr and mass accuracy thresholds as described in the text. A simple target/decoy database approach was used to estimate false positive rates through distraction of random hits and to establish threshold criteria (3) such that <1% false positives were included in the peptide list. An Excel spreadsheet file is available (see supplemental tables) that contains all MCP-required information concerning peptide identifications for more than 30,000 identified peptides.

## RESULTS

To assess the ability to quickly identify proteins of interest from primary tissue samples by subtractive proteins analysis,

we performed experiments using a recently developed LTQ FT hybrid ion trap mass spectrometer and a traditional LCQ Deca XP<sup>Plus</sup> instrument. We analyzed spleen tissues pooled from up to seven C57B1/6 mice and separated by magnetic beads into distinct CD45<sup>-</sup> and CD45<sup>+</sup> cell populations (Fig. 1). CD45 magnetic bead separation has been performed routinely in our laboratory, and we typically observe only a small (1–3%) CD45<sup>-</sup> contamination rate in the CD45<sup>+</sup> cell fraction as determined by flow cytometry analysis (data not shown). In the present study, 1 mg of total cell lysate from CD45<sup>-</sup> or CD45<sup>+</sup> cells was resolved by one-dimensional SDS-PAGE,

TABLE I  
Summary of proteomic analyses of spleen cell samples analyzed by LTQ FT and LCQ XP

	Spleen cell population <sup>a,b</sup>			
	LTQ FT		LCQ XP	
	CD45 <sup>-</sup>	CD45 <sup>+</sup>	CD45 <sup>-</sup>	CD45 <sup>+</sup>
Protein for SDS-PAGE (mg)	1	1	1	1
Gel slices analyzed	14	14	14	14
HPLC gradient time (min)	55	55	55	55
MS scans	7,987	7,554	6,097	6,109
MS/MS scans	78,870	75,539	30,484	30,543
XCorr (1 <sup>+</sup> , 2 <sup>+</sup> , 3 <sup>+</sup> , 4 <sup>+</sup> ) <sup>c</sup>	1, 1, 1, 1	1, 1, 1, 1	1.9, 2, 2.9, N/A	1.9, 2, 2.9, N/A
$\Delta$ Corr <sup>c</sup>	None	None	None	None
ppm cutoff <sup>c</sup>	4.5	4.5	None	None
Unique proteins identified	2,425	1,948	1,147	989
Unique peptides identified <sup>d</sup>	12,175 <sup>d</sup>	12,466 <sup>d</sup>	3,721 <sup>d</sup>	3,823 <sup>d</sup>

<sup>a</sup> One milligram of whole cell protein fraction was prepared from CD45<sup>-</sup> and CD45<sup>+</sup> spleen cells.

<sup>b</sup> A total of 28 gel slices were prepared by in-gel trypsin digestion and analyzed by LTQ FT and LCQ XP over a 55-min HPLC gradient for each sample.

<sup>c</sup> Peptides were identified with SEQUEST (19) using no enzyme specificity. Only fully tryptic peptide matches were considered. Due to two very powerful constraints (search nontryptic  $\rightarrow$  require tryptic; search 1.1 Da  $\rightarrow$  require 4.5 ppm) almost no other thresholding was required to achieve <1% false positives on the LTQ FT. Thresholds for XCorr,  $\Delta$ Corr, and ppm cutoff were determined via a target/decoy database approach (3) to allow for a maximum of 1% false positive rate at the peptide level (see Fig. 4). N/A, not applicable.

<sup>d</sup> See Supplemental Tables 5–8 for a list of peptides identified by LTQ FT and LCQ XP analyses.

and 14 independent in-gel trypsin digests of each cell population were prepared for LC-MS/MS analysis. A total of 28 gel slices were prepared, and a small aliquot was analyzed by both LTQ FT and XP mass spectrometers. Proteins were identified by SEQUEST using only fully tryptic peptides as a starting point for matches. In addition, each dataset was required to have only a 1% false positive rate as estimated via a target/decoy database approach (3). CD45<sup>-</sup> and CD45<sup>+</sup> cell populations were further characterized by a simple subtractive comparison of the proteins identified in each group. Proteins identified by at least two unique peptides were used to create a list of proteins exclusive to each group (CD45<sup>-</sup> and CD45<sup>+</sup>). In addition, we used a gene ontology classification to further characterize proteins exclusive to CD45<sup>-</sup> cells. Approximately 90% of all proteins were associated with at least one gene ontology (as assigned by the gene ontology consortium, [www.geneontology.org](http://www.geneontology.org)) in an automated fashion using the Java application GoMiner (21).

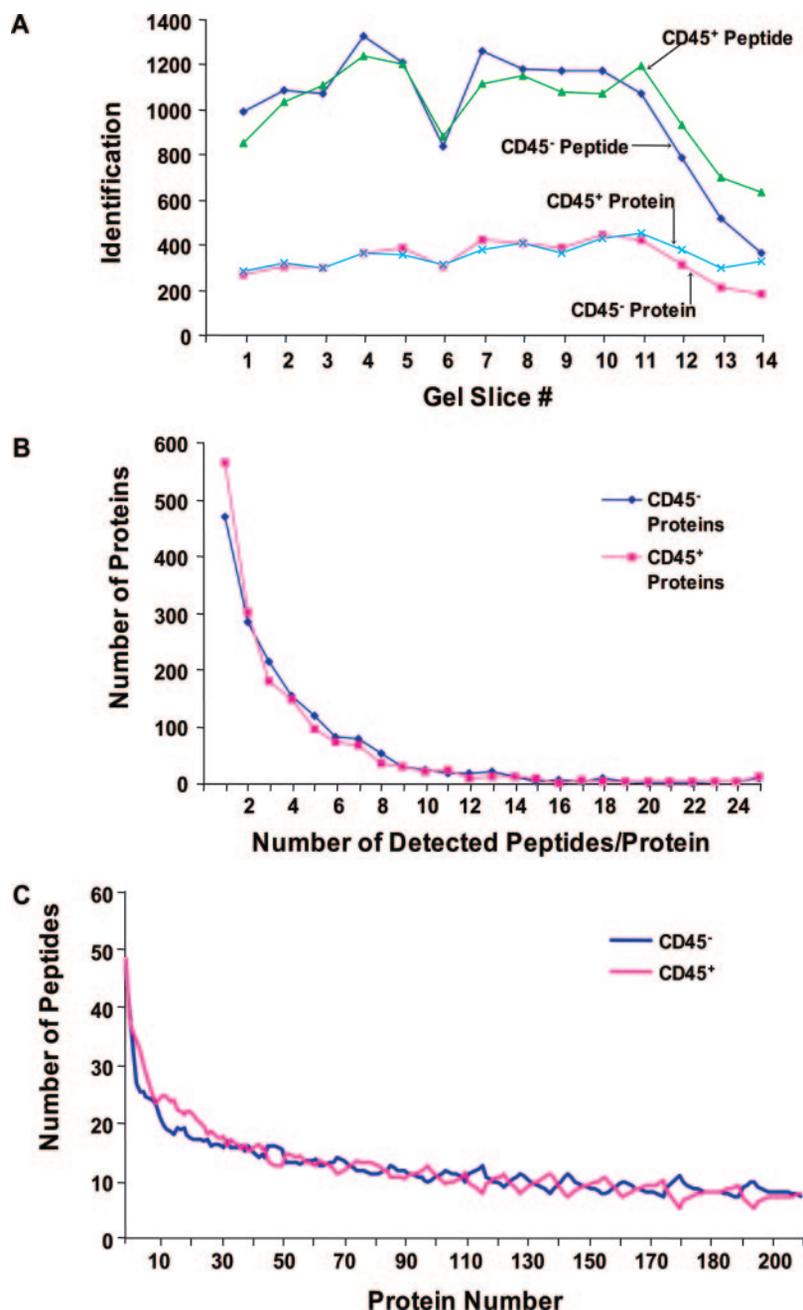
As anticipated the LTQ FT instrument significantly outperformed the more traditional three-dimensional ion trap instrument. Fig. 2A shows a base peak chromatogram for a typical gel slice generated from a CD45<sup>-</sup> sample that was analyzed by both the LTQ FT and LCQ XP under identical columns, sample loading, and gradient conditions. Although both instruments utilize rapid prescans to ameliorate space-charge effects from heterogeneous ion fluxes common to peptide chromatographic separations (termed AGC by the vendor), the feature is particularly important to the LTQ FT in enabling optimization of the number of ions entering the ICR cell for improved mass accuracy (16). In the present studies the AGC was set at a maximum value of  $2 \times 10^6$ . At this AGC setting, the LTQ accumulation time during the analysis varied from 123 ms to a maximum set at 1,200 ms. The average accumu-

lation time was 600 ms, and the average cycle time was 4.3 s for 10 MS/MS scans per cycle (Fig. 2B). The LTQ FT generated ~570 FT-MS spectra and ~5,700 MS/MS spectra from a single gel slice over a 55-min gradient. From the example shown in Fig. 2A, 1,258 unique peptides (421 proteins) were identified (gel slice 7). In contrast, the analysis on the XP acquired five MS/MS scans/cycle with an average scan time of 7.3 s. Approximately 450 MS and 2,300 MS/MS spectra were acquired per gel slice. In the example shown, 442 unique peptides (155 proteins) were identified from gel slice 7.

Table I presents a summary of the entire experiment. In total, 56 samples (28 on each instrument) were analyzed. Database searching using a composite target/decoy database, where mouse proteins were present in both a forward and a reversed orientation (3), was used to estimate false positive rates from each dataset and to provide final filtering criteria. Each dataset was allowed a maximum estimated false positive rate of 1%. The final filtering criteria for the LTQ FT dataset utilized mass accuracy as an additional constraint, which allowed for lower Xcorr values to be used. Although fewer proteins were ultimately identified in the CD45<sup>+</sup> samples, this was not due to sample loading because for most proteins similar numbers of CD45<sup>+</sup> and CD45<sup>-</sup> peptides were detected (Fig. 3). In fact, more total peptides were actually identified in the CD45<sup>+</sup> cells on both the XP and LTQ FT mass spectrometers (Fig. 3). It is likely that this difference comes from the additional complexity of the CD45<sup>-</sup> cells where more than one proteome is represented including that of stem cells.

Fig. 4 shows the effect of mass accuracy on large scale proteomic experiments. Fig. 4A displays all peptide matches from the analysis of a single gel region on the LTQ FT after filtering to only allow fully tryptic matches. True positive matches always come from the forward oriented sequences,

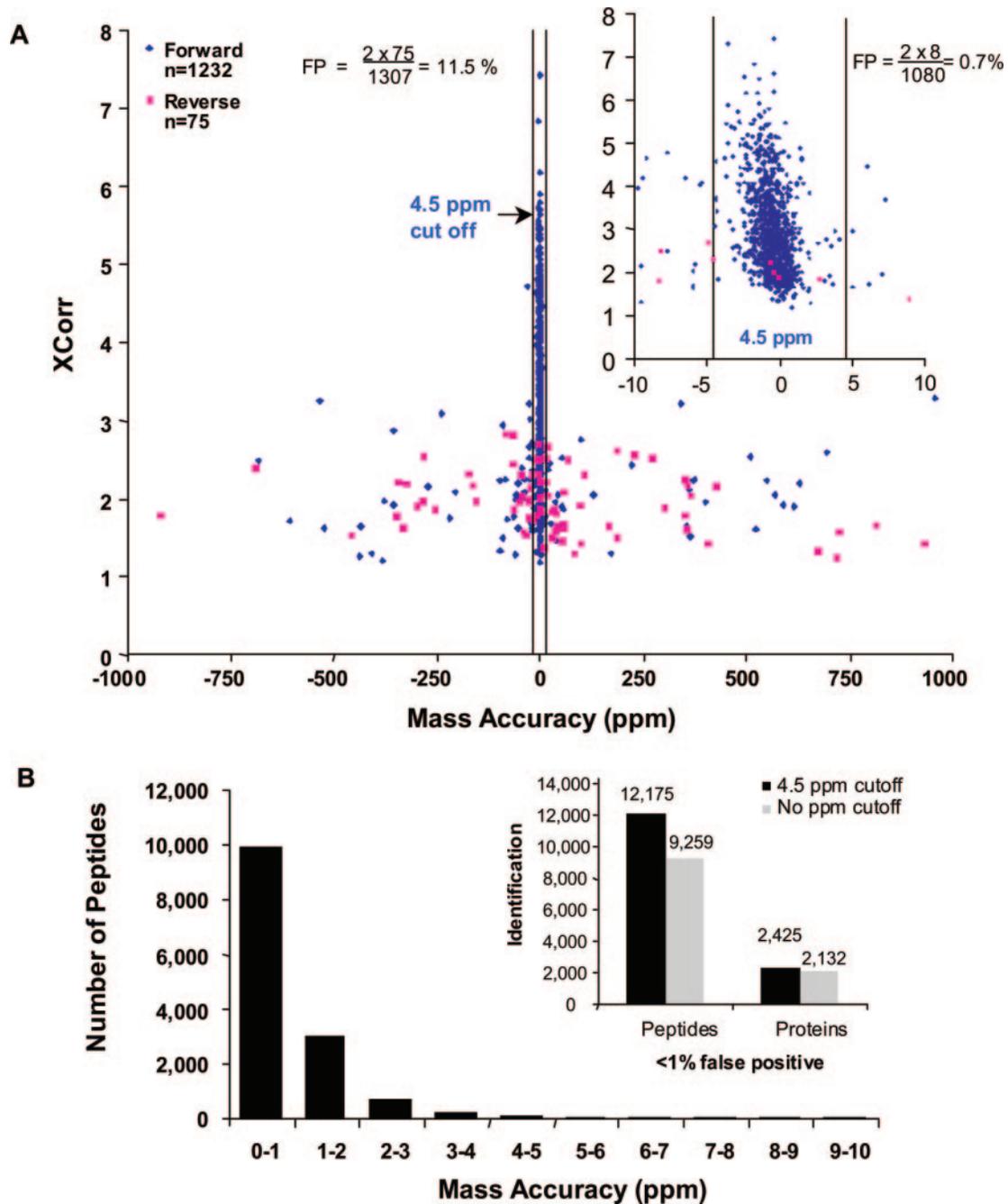
**FIG. 3. Comparison of CD45<sup>-</sup> and CD45<sup>+</sup> proteins detected by LTQ FT.** A, very similar numbers of peptide and protein matches were found for each gel slice. There was a slight increase in the total number of peptides identified in CD45<sup>+</sup> samples prior to filtering for redundancy. B, distribution of protein identifications by number of unique peptides detected per protein. C, comparison of the number of peptides/protein for each of the top 200 most detected proteins from CD45<sup>-</sup> and CD45<sup>+</sup> samples displayed as a running average of the five adjacent proteins.



but false positives are equally split as matches to either orientation. Not using mass accuracy as a filter provided 75 reversed hits of 1,307 total peptides for an unacceptable false positive (FP) rate of 11.5% ( $2 \times \text{reverse hits}/\text{total hits} \times 100\%$ ). Using the mass accuracy filter (4.5 ppm) resulted in a FP rate of only 0.7% (Fig. 4A, *inset*). In an analysis of the peptides identified in all CD45<sup>-</sup> samples combined (14 gel slices) by LTQ FT, ~10,000 peptides had mass accuracies of <1 ppm (Fig. 4B). The distribution of all peptides identified within 10 ppm is shown in Fig. 4B. To contain >99% of the correct answers, 4.5 ppm was used as the final cutoff for correct matches. The average absolute mass deviation was

0.84 ppm for all accepted peptides. It should be noted that only a minimal XCorr cutoff (1.0) was needed when both a tryptic peptide requirement and mass accuracy were used. When a mass accuracy cutoff was not used to identify correct answers, we were required to increase both XCorr and  $\Delta\text{Corr}$  to maintain a false positive rates of <1%, significantly reducing the total number of peptides and proteins identified (Fig. 4B, *inset*).

We next used subtractive proteomics to discover differences between CD45<sup>-</sup> and CD45<sup>+</sup> cell populations. We compared results from both instruments of all identified proteins (all protein hits) and proteins identified by two or more pep-



**FIG. 4. The effect of mass accuracy on peptide identification by LTQ FT analysis.** A, distribution of peptide spectral matches from SEQUEST analysis of an LC-MS/MS analysis of a single representative gel region using a composite target/decoy mouse database. The effect of XCorr and mass accuracy (ppm) is shown. The only requirement was that peptide spectral matches be fully tryptic. Peptide spectral matches derived from the decoy (reversed) database are shown in red, and those derived from the target (forward) database are shown in blue. The estimated FP rate was 11.5% for this dataset. Further filtering for correct matches concentrated in the ppm region between  $\pm 4.5$  ppm resulted in an estimated FP rate of 0.7%. B, mass accuracy (ppm) distribution (between 0 and 10 ppm) for all CD45<sup>-</sup> samples (14 gel regions) analyzed by LTQ FT using only tryptic peptides and an XCorr cutoff of 1.0. The inset shows total unique peptides and proteins in 14 CD45<sup>-</sup> gel regions at <1% false positive peptides detected with and without ppm cutoff. When a ppm cutoff was not used, higher Xcorr and  $\Delta$ Corr were required to maintain a low false positive rate, reducing the total number of peptides and proteins identified.

tides in one sample (excluding one-hit proteins) (Fig. 5). As anticipated, significantly more proteins were identified by the LTQ FT for either all protein hits or proteins identified with two or more peptides per protein. We also observed an  $\sim 10\%$

increase in the number of overlapping proteins between cell groups when using the LTQ FT. In an analysis of the most abundant differences between cell groups (excluding one-hit proteins), relatively few proteins were identified exclusively in

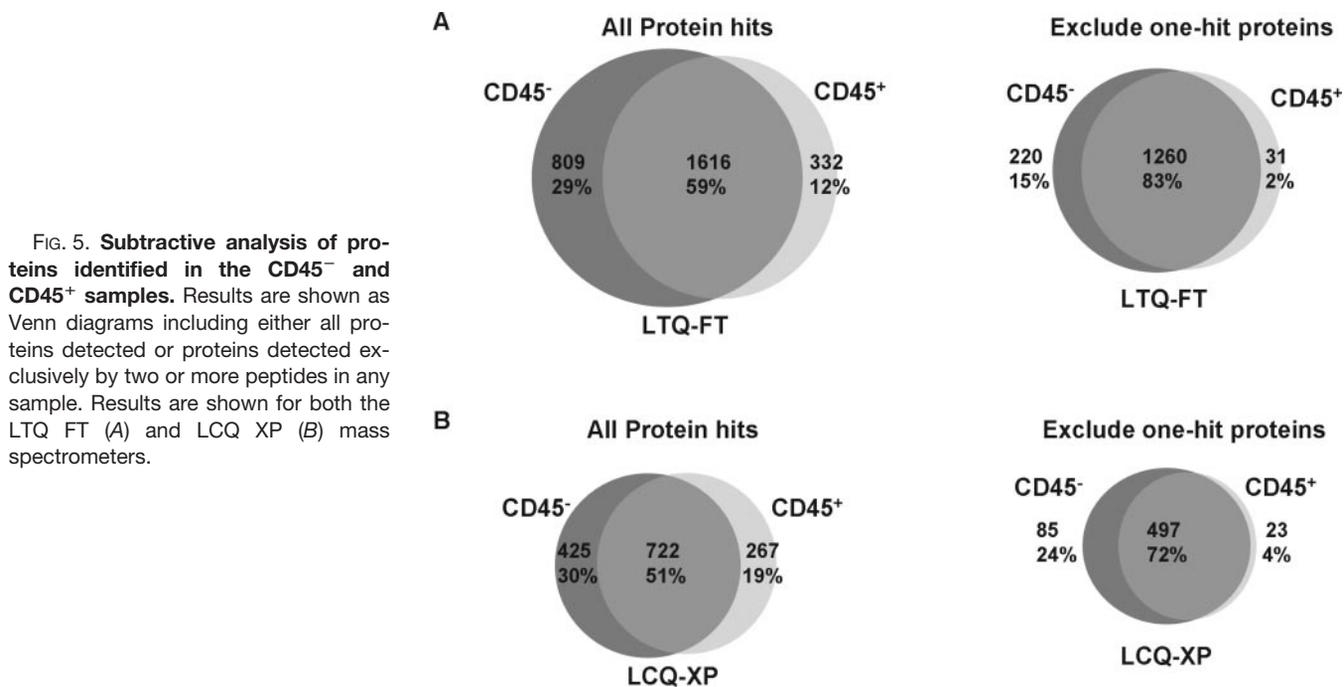


FIG. 5. Subtractive analysis of proteins identified in the CD45<sup>-</sup> and CD45<sup>+</sup> samples. Results are shown as Venn diagrams including either all proteins detected or proteins detected exclusively by two or more peptides in any sample. Results are shown for both the LTQ FT (A) and LCQ XP (B) mass spectrometers.

TABLE II

Comparison of exclusive CD45<sup>-</sup> cell proteins identified by LCQ XP in two independent analyses of 14 gel regions

Proteins exclusively identified in Exp. 1 (XP <sub>1</sub> -CD45 <sup>-</sup> )		Proteins exclusively identified in both Exp. 1 and Exp. 2 (XP <sub>2</sub> -CD45 <sup>-</sup> )	
No. of peptides/protein	No. of proteins from CD45 <sup>-</sup>	No. of proteins identified in both <sup>a</sup>	Percent reproducible <sup>b</sup>
7+	4	2	50
6	1	1	100
5	1	0	0
4	3	1	33
3	15	4	27
2	62	20	32
1	340	59	17

<sup>a</sup> Protein identifications in experiment 2 were exclusive but not necessarily identified by the same number of peptides.

<sup>b</sup> For results to be considered reproducible proteins had to be identified in replicated studies and identified as exclusive to CD45<sup>-</sup>.

TABLE III

Comparison of proteins identified by subtractive proteomics in CD45<sup>-</sup> samples in LTQ FT in two independent analyses of 14 gel regions

Proteins exclusively identified in Exp. 1 (FT <sub>1</sub> -CD45 <sup>-</sup> )		Proteins exclusively identified in both Exp. 1 and Exp. 2 (FT <sub>2</sub> -CD45 <sup>-</sup> )	
No. of peptides/protein	No. of proteins from CD45 <sup>-</sup>	No. of proteins identified in both <sup>a</sup>	Percent reproducible <sup>b</sup>
8+	6	5	83
6	7	5	71
5	8	4	50
4	20	10	50
3	30	13	43
2	149	66	44
1	589	173	29

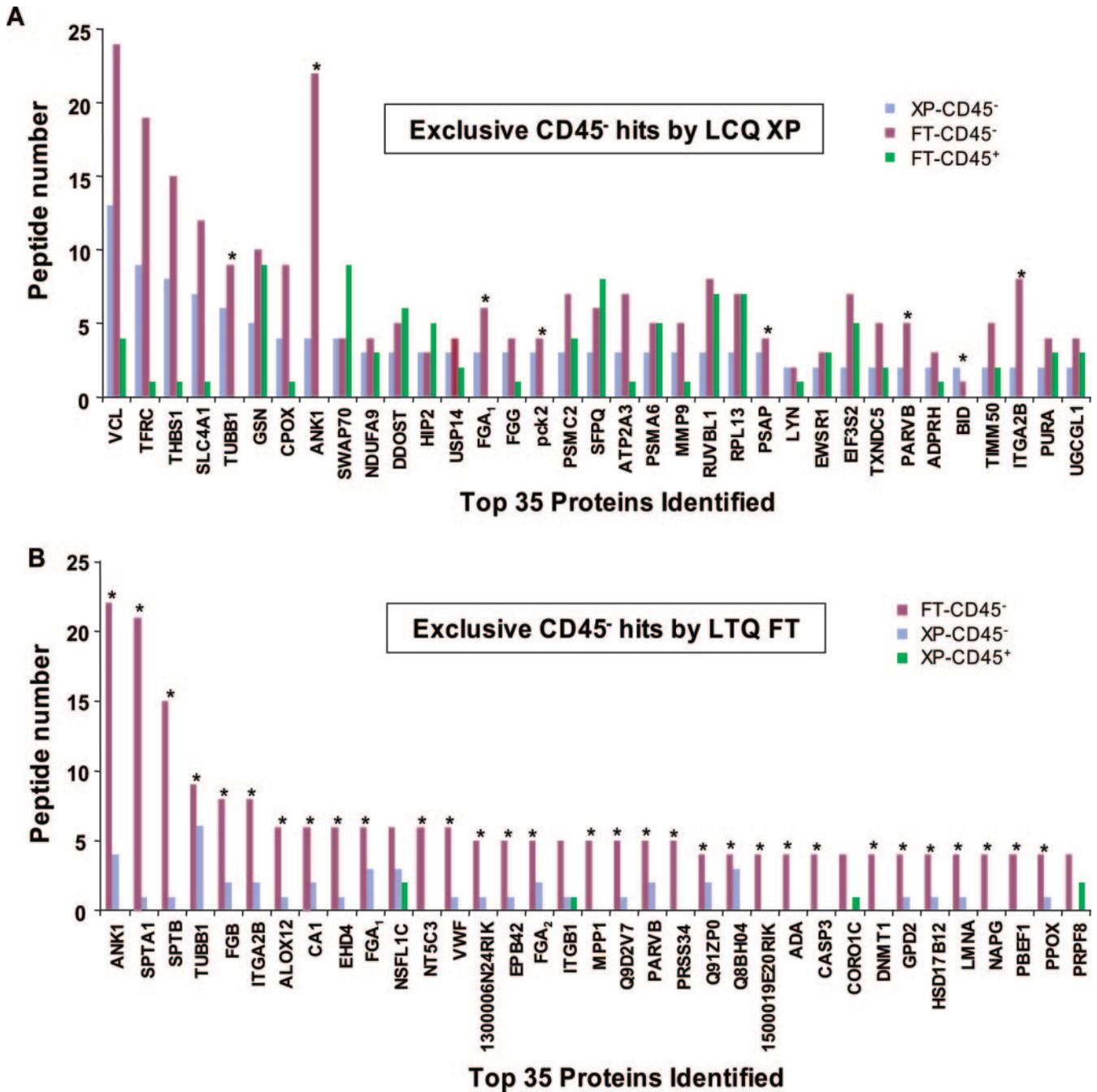
<sup>a</sup> Protein identifications in experiment 2 were exclusive but not necessarily identified by the same number of peptides.

<sup>b</sup> For results to be considered reproducible proteins had to be identified in replicated studies and identified as exclusive to CD45<sup>-</sup>.

CD45<sup>+</sup> samples by both the LTQ FT (31 proteins) and the LCQ XP (23 proteins) likely due to the higher purity of that preparation (Fig. 3C). Although the potential biological significance of the differences in proteins identified in spleen cells will be presented elsewhere, a preliminary description of the 220 and 31 most abundant proteins exclusively identified in CD45<sup>-</sup> and CD45<sup>+</sup> cells, respectively, is presented in Supplemental Tables 1 and 2.

In an effort to better determine the reproducibility of shotgun subtractive proteomics, we compared all exclusive CD45<sup>-</sup> proteins identified by the XP and LTQ FT from two independent analyses by number of peptides per protein (Tables II and III). The entire experiment including harvesting, SDS-PAGE, gel region excision, and LC-MS/MS analysis was

repeated as the first experiment. Exclusive CD45<sup>-</sup> cell proteins obtained in experiment 1 (XP<sub>1</sub>-CD45<sup>-</sup>) by subtractive analysis were listed by the number of peptides identified for each protein and compared with experiment 2 (XP<sub>2</sub>-CD45<sup>-</sup>). Table II shows that few exclusive CD45<sup>-</sup> cell proteins were identified by XP analysis by four or more peptides. With such low numbers it was difficult to estimate the reproducibility of the results in a replicate XP analysis of the samples. With proteins identified by two to four peptides, ~30% were also identified by subtractive analysis in experiment 2 (XP<sub>2</sub>-CD45<sup>-</sup>). Exclusive CD45<sup>-</sup> cell proteins identified by only one peptide were observed in a replicate analysis only 17% of the time (59 of 340 proteins) with many of the proteins not identified in either CD45<sup>-</sup> or CD45<sup>+</sup> cells. A similar comparison of



**FIG. 6. Comparison of exclusive CD45<sup>-</sup> cell proteins characterized by replicate LCQ XP and LTQ FT studies.** *A*, the top 35 proteins identified by LCQ XP in CD45<sup>-</sup> samples in experiment 1 (XP<sub>1</sub>-CD45<sup>-</sup>) that were exclusive to CD45<sup>-</sup> cells (not present in CD45<sup>+</sup> samples) are compared in two completely independent analyses. Replicate LC-MS/MS analysis showed 17 of the top 35 proteins that were exclusive to CD45<sup>-</sup> samples in XP<sub>1</sub>-CD45<sup>-</sup> were identified as exclusive to CD45<sup>-</sup> samples or not detected in either sample in experiment 2 (XP<sub>2</sub>-CD45<sup>-</sup> and XP<sub>2</sub>-CD45<sup>+</sup>). Of the 18 proteins that were identified in CD45<sup>+</sup> samples in experiment 2, seven proteins were identified with less total unique peptides in CD45<sup>+</sup> samples, indicating that the relative abundance is higher in CD45<sup>-</sup> samples although not exclusive. *B*, the top 35 proteins identified by LTQ FT in CD45<sup>-</sup> samples (FT<sub>1</sub>-CD45<sup>-</sup>) exclusive to CD45<sup>-</sup> samples were compared in two independent analyses. LC-MS/MS analysis of replicate samples by LTQ FT showed 24 of the top 35 proteins that were exclusive to CD45<sup>-</sup> samples in FT<sub>1</sub>-CD45<sup>-</sup> were also exclusive in FT<sub>2</sub>-CD45<sup>-</sup> samples or were not detected in either sample in experiment 2. Of the 11 proteins that were not exclusive in experiment 2, eight were identified with less total unique peptides in CD45<sup>+</sup> samples. ANK1, ankyrin 1; VWF, von Willebrand factor; FGB, fibrogen B-β chain; EHD4, EH domain-containing protein 4; PPOX, protoporphyrinogen oxidase; CA1, carbonic anhydrase I; FGA, fibrogen A-α chain; PARVB, β-parvin; ALOX12, arachidonate 12-lipoxygenase; CASP3, caspase 3; PBEF1, pre-B-cell colony-enhancing factor; LMNA, lamin A; TUBB1, β tubulin 1; SPTA1, spectrin α chain; SPTB, spectrin β chain; MPP1, murine proliferation-associated protein 1; ADA, adenosine-deaminase; NAPG, γ-soluble NSF attachment protein.

TABLE IV  
Characterization of exclusive CD45<sup>-</sup> cell proteins identified by subtractive proteomics

Gene <sup>a,b</sup>	CD45 <sup>-</sup> /CD45 <sup>+</sup> <sup>c</sup>	Protein name	Function
ANK1	22/0	Ankyrin 1	Attach integral membrane proteins to cytoskeletal elements
SPTA1	21/0	Spectrin $\alpha$ chain, erythrocyte	Cytoskeletal structure erythrocyte plasma membrane
SPTB	15/0	Spectrin $\beta$ chain, erythrocyte	Cytoskeletal structure erythrocyte plasma membrane
TUBB1	9/0	$\beta$ tubulin 1, class VI	Microtubule polymerization
FGF	8/0	Fibrinogen B- $\beta$ chain	Cofactor in platelet aggregation
ITGA2B	8/0	Integrin $\alpha$ -IIb precursor	Receptor for fibronectin involved in platelet aggregation
CA1	6/0	Carbonic anhydrase I	Reversible hydration of carbon dioxide
VWF	6/0	von Willebrand factor	Promotes adhesion of platelets to the sites of vascular injury
EHD4	6/0	EH domain-containing protein 4	Unknown
ALOX12	6/0	Arachidonate 12-lipoxygenase	Has oxygenase and 14,15-leukotriene A4 synthase activity
PARVB	5/0	$\beta$ -Parvin	Regulation of cell adhesion and cytoskeleton organization
FGA	5/0	Fibrogen A- $\alpha$ chain	Involved in platelet aggregation
EPB42	5/0	Erythrocyte membrane protein band	Regulation of erythrocyte shape
PRSS34	5/0	Mast cell protease-11	Unknown; possible serine-type peptidase activity
LMNA	4/0	Lamin A	Component of the nuclear lamina
PSAP	4/0	Sulfated glycoprotein 1	Unknown
PPOX	4/0	Protoporphyrinogen oxidase	Catalyzes the six-electron oxidation of protoporphyrinogen IX
PBEF1	4/0	Pre-B-cell colony-enhancing factor	Unknown
CASP3	4/0	Caspase 3	Activation cascade of caspases responsible for apoptosis execution
PSMB5	4/0	Proteasome subunit $\beta$ type 5	The proteasome has an ATP-dependent proteolytic activity
NAPG	4/0	$\gamma$ -Soluble NSF <sup>d</sup> attachment	Required for vesicular transport between the ER <sup>e</sup> and the Golgi
Q91ZP0	4/0	Fibrinogen $\gamma$ -chain	Cofactor in platelet aggregation
XRN2	3/0	5'-3' exoribonuclease 2	Possesses 5'-3' exoribonuclease; involved in homologous recombination
AP2M1	3/0	Clathrin coat assembly protein AP50	Component of the adaptor complexes that link clathrin to receptors
FECH	3/0	Ferrochelatase	Catalyzes the ferrous insertion into protoporphyrin IX

<sup>a</sup> Top 25 proteins identified by subtractive proteomics by LTQ FT analysis.

<sup>b</sup> Final list of genes expressed in CD45<sup>-</sup> cells was produced from exclusive CD45<sup>-</sup> genes identified in LTQ FT experiment 1 compared to a replicate LTQ FT study and two replicated LCQ XP analyses.

<sup>c</sup> Total number of unique peptides identified for each gene in CD45<sup>-</sup> and CD45<sup>+</sup> cells in LTQ FT experiment 1.

<sup>d</sup> N-Ethylmaleimide-sensitive factor.

<sup>e</sup> Endoplasmic reticulum.

LTQ FT results showing exclusive CD45<sup>-</sup> cell proteins identified in experiment 1 (FT<sub>1</sub>-CD45<sup>-</sup>) with results obtained in experiment 2 (FT<sub>2</sub>-CD45<sup>-</sup>) showed improved reproducibility and many more proteins identified (Table III). As expected, reproducibility decreased with decreasing number of peptides identified. Fig. 6 shows a partial list of exclusive CD45<sup>-</sup> cell proteins characterized by replicate LCQ XP and LTQ FT studies.

Using the LTQ FT, we identified 220 proteins by two or more peptides exclusively in CD45<sup>-</sup> cells through subtractive proteomics. As a preliminary assessment and comparison of the performance of the two instruments only exclusive proteins identified by LTQ FT analysis in experiment 1 were characterized using a Swiss-Prot/TrEMBL/UniProt database search ([us.expasy.org](http://us.expasy.org) and [www.pir.uniprot.org](http://www.pir.uniprot.org)). A list of the 220 proteins is shown in Supplemental Table 1 by the number of peptides identified for each protein in LTQ FT experiment 1. The number of peptides identified in subsequent analysis by LTQ FT and LCQ XP is also shown. Of the 220 exclusive proteins identified in LTQ FT experiment 1, 126 proteins were consistently absent in CD45<sup>+</sup> cell samples after repeated analysis. A partial list of the most abundant protein differences

identified in CD45<sup>-</sup> cells that were consistent across studies is shown in Table IV. Some of the most abundant differences observed between cell types were due to a small population of erythrocyte and platelet cells. Red blood cells are the dominant cell type in CD45<sup>-</sup> cell fractions within the spleen population, and although most (>98%) are removed during the initial cell lysis of spleen tissue, small amounts remained. Platelets also do not express CD45 and were identified within the CD45<sup>-</sup> cell fraction, and although they are likely not involved in pancreatic cell regeneration they provided a useful marker that demonstrates the ability of subtractive proteomic analysis to identify exclusive CD45<sup>-</sup> proteins. Relatively few (thirty-one) exclusive proteins were identified in CD45<sup>+</sup> samples by LTQ FT. A list of exclusive CD45<sup>+</sup> cell proteins from FT experiment 1 and the consistency of peptide identification over multiple studies are shown in Supplemental Table 2.

Gene ontology characterization using the Javascript GoMiner (21) provided a way to further categorize these identified gene products by function. Of particular interest to this study were stem cell proteins that might be involved in the regeneration process of pancreatic cells already described (18). GoMiner was used to apply all known gene ontology

categories associated with the 126 proteins exclusive to CD45<sup>-</sup> spleen cells in an automated fashion. Gene ontology terms (as defined by the gene ontology consortium, [www.geneontology.org/](http://www.geneontology.org/)) were assigned to ~90% of the proteins identified by subtractive proteomic analysis. A partial list of spleen cell proteins described by seven different gene ontology terms (cell adhesion, cell cycle, development, DNA binding, extracellular space, integral to membrane, and signal transduction) that may identify relevant CD45<sup>-</sup> spleen cell proteins is shown in Supplemental Table 3. Because we were investigating the ability of CD45<sup>-</sup> cells to regenerate pancreatic tissue, we focused on proteins involved in development and other related gene ontology terms. Of the 126 exclusive proteins 24 were identified as being derived from platelet or blood cells and were excluded from further gene ontology characterization. Of the remaining 112 exclusive proteins, 49 were characterized by the gene ontology terms described above, and 14 proteins not associated with any gene ontology terms are also described (Supplemental Table 3). The remaining 49 proteins were not identified by any of the seven gene terms of interest. Of particular interest for future analysis and characterization are seven proteins involved in development. Another interesting observation is that ~50 of the proteins described in Supplemental Table 3 (shaded proteins) have been observed previously in mouse tissue at various stages of development, and many proteins are involved in spermatogenesis and other biological processes relevant to stem cell biology ([www.informatics.jax.org/](http://www.informatics.jax.org/)). A list of all the gene ontology terms associated with the 126 exclusive CD45<sup>-</sup> proteins is shown in Supplemental Table 4.

#### DISCUSSION

A recent comparison between human and mouse genomes showed gene homology approaching 99% (22), indicating that the diversity between such far removed mammals is largely dependent on differences in protein regulation and expression of strikingly similar genes. This further highlights the importance of developing tools to evaluate biological diversity at a more global protein level. To meet this challenge, new methods and instrumentation are being developed for a more comprehensive, systems-level understanding of biology. In the current studies we used new instrumentation (LTQ FT) to characterize a complex protein mixture by subtractive proteomics. In preparations of whole cell fractions from two diverse spleen cell populations we identified ~2,000 proteins per preparation from more than 12,000 peptides by LTQ FT analysis and, by comparison, ~1,000 proteins (~3,700 peptides) by LCQ XP analysis. Each of these peptide datasets had a false positive rate estimated to be <1% based on a target/decoy database searching strategy (3). A subtractive proteomic comparison of proteins expressed by CD45<sup>-</sup> and CD45<sup>+</sup> cells was also improved by replicating the entire experiment.

The field of quantitative proteomics provides a discipline for differential protein expression profiling. Stable isotope label-

ing is the surest way to precisely measure differences in protein abundance. However, the object of some proteomic experiments is to identify proteins that are exclusively expressed in one state but not the other. Two recent studies have provided useful experience with subtractive proteomics as a quantitative proteomic technique (14, 15). Our interest was in finding stem cell-specific proteins that were exclusively expressed in the CD45<sup>-</sup> cell population. Some evidence of the usefulness of the technique comes from the finding of red blood cell- and platelet-specific proteins exclusively in the CD45<sup>-</sup> cells. This is because a very small amount of red blood cells and platelets are not completely removed during cell preparations and remain within CD45<sup>-</sup> cell populations. Twenty-four proteins of 126 from subtracted CD45<sup>-</sup> datasets were either red blood or platelet cell proteins.

We believe the idea of using shotgun sequencing of whole proteomes followed by subtractive analysis will be useful for many specific proteomic applications because it (i) can be used on primary tissue, (ii) makes use of mature technologies (peptide sequencing) that require little refinement, (iii) can be improved by simple replication of the experiment to provide more significant differences, and (iv) relies on instrumentation that now can provide much deeper analysis of protein sequences because of increased scan rates and mass accuracy.

One stated purpose of this project was to initiate the protein profiling of a newly identified stem cell population in the spleen of adult mice (18). This stem cell population is contained within the capsular regions of the CD45<sup>-</sup> regions of the spleen and has been proposed to be a remnant of an embryonic stem cell region called the aorta gonad mesoderm (23). Mass spectrometry analysis of the CD45<sup>-</sup> cell subset readily revealed proteins involved in development, indicative of the persistence of a fetal stem cell in an adult animal. Subtractive proteomics showed development-specific proteins that control the formation of the fetal nervous system (cerebellum, neurogenesis, and axon guidance axonogenesis), blood vessels, muscle, skin, and gonads (gametogenesis and spermatogenesis). Although many of the proteins exclusive to CD45<sup>-</sup> samples had unknown function, the majority are abundantly expressed on day 10–11 of gestation (Supplemental Table 1). Therefore, this preliminary analysis of the CD45<sup>-</sup> fractions of the spleen is consistent with a stem cell population that might represent a frozen fetal cluster of a midstage murine embryo (24). These interesting candidate proteins were (i) identified by two or more unique peptides, (ii) exclusive to CD45<sup>-</sup> samples, (iii) detected by LTQ FT analysis, and (iv) consistent across replicate studies. These results indicate that the improved performance of the LTQ FT significantly aided our ability to characterize complex protein mixtures by shotgun proteomics. Our primary focus for future analysis will be proteins implicated in development and exclusive integral plasma membrane proteins that maybe used to more specifically isolate potential stem cells from the CD45<sup>-</sup> cell population.

Shotgun proteome analysis by LC-MS/MS with new instru-

mentation provided significantly larger datasets for characterizing protein expression differences. Yet a number of concerns remain in particular with respect to the reproducibility of the results and with data processing. The reproducibility was considerably improved by LTQ FT analysis (Table III) but remained low for the majority of the proteins, which were typically identified by five or less peptides. This can be somewhat misleading for a truly subtractive approach. It is likely that many proteins are expressed differentially between the two populations but not exclusively. If a 10-fold difference in protein expression results in a peptide being detected in the control sample of one replicate but not in the other, this protein would not be included in the subtracted list but may be of great interest to the investigator due to the 10-fold difference. Repeated sample analysis can be used to provide more confidence that the protein is truly not present in a cell population or that it is present at significantly less abundant quantities. To improve the reliability of our final data we only accepted subtractive differences that were consistent in multiple analyses.

Gene ontology classification of the proteins identified was used to characterize proteins in an automated fashion to improve data processing. In the present study most of the proteins were associated with at least one gene ontology term, but ~10% were completely unknown. Furthermore the evidence for term assignments vary by protein, and because many proteins have unknown functions, the gene ontology database is incomplete. Studies to characterize the biological relevance of differences observed in these experiments are currently being performed.

**Acknowledgments**—We thank members of the Gygi and Faustman laboratories for fruitful discussions.

\* This work was funded by National Institutes of Health Grants HG003456 (to S. P. G.) and TgT32DK07028 (to D. F.) and by the Iacocca Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at <http://www.mcponline.org>) contains supplemental material.

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