Eight-channel iTRAQ Enables Comparison of the Activity of Six Leukemogenic Tyrosine Kinases*[S]

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There are a number of leukemogenic protein-tyrosine kinases (PTKs) associated with leukemic transformation. Although each is linked with a specific disease their functional activity poses the question whether they have a degree of commonality in their effects upon target cells. Exon array analysis of the effects of six leukemogenic PTKs (BCR/ABL, TEL/PDGFRβ, FIP1/PDGFRα, D816V KIT, NPM/ALK, and FLT3ITD) revealed few common effects on the transcriptome. It is apparent, however, that proteome changes are not directly governed by transcriptome changes. Therefore, we assessed and used a new generation of iTRAQ tagging, enabling eight-channel relative quantification discovery proteomics, to analyze the effects of these six leukemogenic PTKs. Again these were found to have disparate effects on the proteome with few common targets. BCR/ABL had the greatest effect on the proteome and had more effects in common with FIP1/PDGFRα. The proteomic effects of the four type III receptor kinases were relatively remotely related. The only protein commonly affected was eosinophil-associated ribonuclease 7. Five of six PTKs affected the motility-related proteins CAPG and vimentin, although this did not correspond to changes in motility. However, correlation of the proteomics data with that from the exon microarray not only showed poor levels of correlation between transcript and protein levels but also revealed alternative patterns of regulation of the CAPG protein by different oncogenes, illustrating the utility of such a combined approach. ***Molecular & Cellular Proteomics 7: 853–863, 2008.***

Human leukemias are often associated with chromosome translocations that generate a fusion transcript that can be implicated in disease pathogenesis (for a review, see Ref. 1). Many of these translocation gene products encode for constitutively activated protein-tyrosine kinases (PTKs).[1] These include BCR/ABL (2), a PTK associated with chronic myeloid leukemia, and TEL/PDGFRβ (3) observed in some cases of chronic myelomonocytic leukemia. *FIP1/PDGFRα* fusion gene is associated with the pathogenesis of about 50% of patients with the hypereosinophilic syndrome (4). A t(2, 5) translocation results in the fusion kinase NPM/ALK found in cases of anaplastic large cell lymphoma (5). Recently other forms of PTK dysregulation have been observed in myeloproliferative disorders and the leukemias: the KIT receptor PTK undergoes activating mutations (D816V) in acute myeloid leukemia (6) and in solid tumors; internal tandem duplication at the juxtamembrane region of the *FLT3* gene is a somatic change detected in 20% of cases of acute myeloid leukemia that leads to enhanced ligand-independent PTK activity in the FLT3 internal tandem duplication (ITD) form of the protein (7).

Targeted inhibition of the PTKs (such as KIT, BCR/ABL, and TEL/PDGFRβ) via inhibitors that bind the ATP pocket of the kinase domain has been a successful strategy to achieve hematologic or molecular remission (8). In chronic myeloid leukemia the use of imatinib, a BCR/ABL kinase inhibitor, has profoundly altered approaches to treatment (9). However, after 5 years a significant proportion of patients develop a resistance to the drug, although other molecular therapies remain effective (10). The requirement for further developments in treatment of this and other leukemias is apparent, and further detail of mechanisms for oncogenic tyrosine kinase-mediated leukemic transformation is required.

The development of systems biology offers new opportunities for the study of oncogenic effects of genes deregulated in primary cells from patients with leukemia. Investigation of protein levels in such cells is important because, in hematopoietic cells, changes in the levels of mRNA do not necessarily act as a predictor for changes in the proteome (11). The effects of the TEL/PDGFRβ oncogene on a hematopoietic cell

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; PDGFR, platelet-derived growth factor receptor; iTRAQ, isobaric tags for relative and absolute quantitation; ITD, internal tandem duplication; MSCV, murine stem cell virus; SCX, strong cation exchange; HMGB2, high mobility group protein B2.

line proteome have been shown to be driven by altered transcription plus post-translational regulation of protein levels (12). However, sensitive protein expression analysis has been problematic because amplification of protein, unlike DNA or RNA, cannot be achieved. Relative quantification proteomics using higher sensitivity mass spectrometry techniques now offers the potential to compare and contrast the effects of leukemogenic PTKs. Currently an isobaric tag-based methodology for peptide relative quantification (iTRAQ) coupled to multidimensional liquid chromatography and tandem mass spectrometry enables the assessment of protein levels where four samples can be compared for their common effects (13). Here we report the use of a novel eight-channel relative quantification mass spectrometry technique to compare the relative effects of six oncogenic tyrosine kinases, BCR/ABL, TEL/PDGFRβ, FIP1/PDGFRα, D816V KIT, NPM/ALK, and FLT3ITD. The effects of the oncogenes on the proteome are distinctive and do not follow normal oncogene classification patterns.

EXPERIMENTAL PROCEDURES

Cell Lines—Ba/F3 cells were transfected with either an empty MSCV retroviral vector or MSCV containing *BCR/ABL*, *TEL/PDGFRβ*, *FIP1/PDGFRα*, *D816V KIT*, *NPM/ALK*, or *FLT3ITD*. The resultant cell lines were maintained in culture in Fischer's medium (Invitrogen) with 10% (v/v) horse serum (Biowest, Nuaillé, France). Ba/F3 MSCV cells were grown in Fischer's medium with 10% (v/v) horse serum supplemented with 5% mIL-3 (conditioned medium from X63-Ag-653 cells).

Microarray Analysis—RNA was prepared using TRIzol (Invitrogen), DNase-treated, and then cleaned using MinElute RNeasy cleanup kit (Qiagen) according to the manufacturers' instructions. Transcriptome analysis was undertaken using murine GeneChip® Mouse Exon 1.0 ST Arrays by the CR-UK Affymetrix microarray facility (Paterson Institute, Manchester, UK). All data were analyzed in R using Bioconductor (14). Expression summaries were generated using robust multiarray average (RMA) (15) with a custom chip definition file produced using the makecdfenvs library.[2] Fine grained gene level annotation was provided using the X:MAP database and the exonmap BioConductor package (16). It is unreasonable to assume that a given gene should have the same base-line level of expression in each of the different cell lines or that the same level of response should be observed across all cell lines. Consequently it is unreasonable to expect either the expression levels or -fold changes to come from a single distribution. Thus each cell line was analyzed separately, and the set of probe sets exceeding a 2-fold change was selected. Up- and down-regulated probe sets were treated separately. For each probe set, the number of cell lines in which it was found to be up- or down-regulated was determined and used to provide a score. Data were permuted to create a null distribution of these scores from which the false discovery rate (17) was estimated. The sets of probe sets found to be consistently differentially expressed in six, five, or four oncogene-expressing cell lines were identified and then filtered using exonmap to exclude those that did not hit within an Ensembl (18) annotated gene or that hit the genome at more than one location. Remaining probe sets were then mapped via exonmap to their target genes.

Liquid Chromatography and Mass Spectrometry—Cell pellets were produced and processed as described previously (12) with minor modifications. In brief, cells were lysed in 1 M triethylammonium bicarbonate with 0.1% (w/v) SDS. 50 μg of protein from each cell line was reduced, alkylated, and subjected to tryptic hydrolysis prior to labeling with eight-channel iTRAQ reagent (Applied Biosystems, Framingham, MA) in 1 M triethylammonium bicarbonate according to the manufacturer's instructions (in all experiments labeling exceeded 98% of total identified peptides). Two biological replicates of MSCV Ba/F3 cells were labeled to provide an internal control for the experiment and assess biological variation. The entire experiment (including generation of cell pellets) was performed twice. Peptides were fractionated off line using an SCX column (40 fractions) prior to reverse phase LC-MS/MS using a QStar XL (Applied Biosystems). Data were acquired using an independent data acquisition protocol where an MS scan was taken, and then the two highest abundance (ion current) ions were selected for fragmentation followed by dynamic exclusion for 1 min.

Data Analysis—Data were processed by a "thorough" search against a mouse Celera Discovery Systems database (mouse_K-BMS5_0_20050302, 115,660 entries) using the Paragon algorithm (19) within ProteinPilot version 2.0 software with trypsin as the digest agent and default settings (Applied Biosystems, Warrington, UK). This software calculates a percentage of confidence that reflects the probability that the hit is a false positive so that at the 95% confidence level there is a false positive identification rate of around 5% (19). Although this software automatically accepts all peptides with a confidence of identification >1%, only proteins that had at least one peptide with >95% were initially recorded. These low confidence peptides therefore do not identify a protein by themselves but may support the presence of a protein identified using other peptides. Performing the search against a concatenated database containing both forward and reversed sequences (therefore 231,320 entries) allowed estimation of the false discovery level.

For protein relative quantification, only MS/MS spectra that were unique to a particular protein and where the sum of the signal-to-noise ratio for all of the peak pairs was >9 were used for quantification (default software settings). The accuracy of each protein ratio is given by a calculated "error factor" in the software, and a p value is given to assess whether the protein is significantly differentially expressed. The error factor is calculated as $10^{(95\%\ \text{confidence error})}$ where this 95% confidence error is the weighted standard deviation of the weighted average of log ratios multiplied by Student's t factor for $n - 1$ degrees of freedom where n is the number of peptides contributing to protein relative quantification. The p value is determined by calculating Student's t factor by dividing (weighted average of log ratios − log bias) by the weighted standard deviation, allowing determination of the p value with $n - 1$ degrees of freedom again where n is the number of peptides contributing to protein relative quantification. For the identification of expression differences, each experimental run was initially considered separately. To be identified as being significantly differentially expressed, a protein had to be quantified with at least three spectra (allowing generation of a p value), have a p value <0.05, and have a ratio -fold change >1.2 or <0.8 in both experimental replicates. These -fold change limits were selected on the basis of our previous work with the fourplex iTRAQ reagent (12) and were shown here to be equally applicable to the eightplex iTRAQ relative quantification by the inclusion of internal controls (see Fig. 2*D*). Data were further analyzed using the Genespring GX microarray analysis package (Agilent Technologies).

Western Blotting and Activity Assays—Western blotting was performed using standard protocols. Antibodies used were actin (1:500; Sigma); CAPG (0.25 μg/ml; Abcam, Cambridge UK); ABL, FLT3, KIT, and PDGFRα (1, 0.1, 1, and 1 μg/ml, respectively; Santa Cruz Biotechnology, Inc., Calne, UK); phosphotyrosine 4G10 (50 ng/ml; Up-

[2] R. A. Irizarry, L. Gaultier, W. Huber, and B. Bolstad, unpublished data.

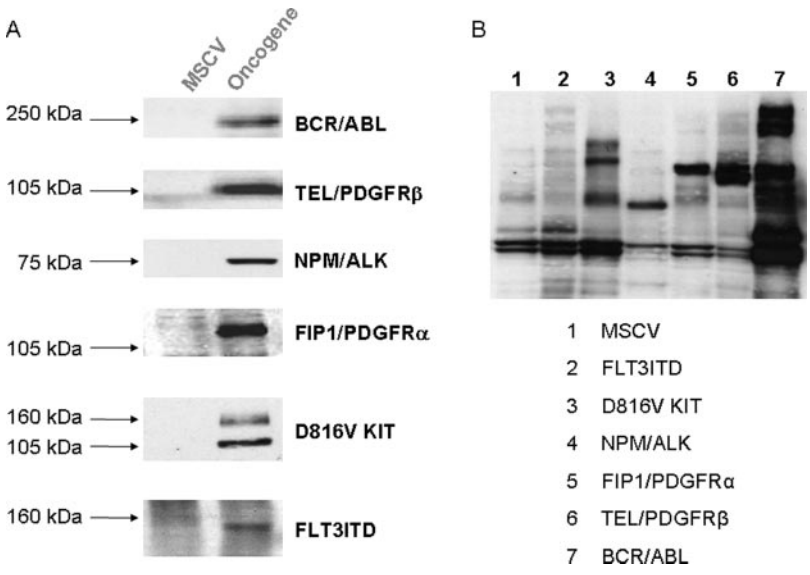

FIG. 1. **Retroviral expression of oncogenes in Ba/F3 cells.** Ba/F3 cells were transfected with MSCV retroviral vector containing the different oncogenes as indicated. *A*, following drug selection the successful transfection was confirmed by assessing oncogene expression by Western blot analysis on whole cell lysates. *B*, the effect of oncogene expression on the phosphotyrosine protein content of the cell was assessed by Western blot analysis with anti-phosphotyrosine antibody.

state, Milton Keynes, UK); PDGFRβ (1 μg/ml; BD Pharmingen); and NPM (neat hybridoma supernatant, LRF Diagnostics Unit, Oxford, UK).

Quantitative Real Time PCR Analysis—Primers and probes were designed using the Universal Probe Library Assay Design Centre from Roche Applied Sciences. QRT-PCR assays were performed in triplicate on a 7900HT Fast Real-Time PCR System. ΔΔCt values were calculated for each sample against the average of the two housekeeping genes that were used to calculate -fold change using the $2^{-\Delta\Delta Ct}$ method (20).

Chemotaxis Assays—Chemotaxis assays were performed as described previously (21) except that serum-free StemSpan medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) was used to preclude oncogene-mediated responses to serum-associated agonists, and assays were carried out in 24 Transwell plates with 5-μm inserts (Sigma).

RESULTS

A Common Cell Population for Relative Analysis of PTK Effects—The role of tyrosine kinases in leukemogenesis is undisputed. The panoply of kinases involved suggests there could be common downstream targets, and our aim was to systematically analyze several leukemogenic protein-tyrosine kinases. Six different leukemogenic PTKs were expressed in a common Ba/F3 background for this purpose (Fig. 1*A*). Ba/F3 cells were chosen because of their widespread usage in studies on leukemogenesis. Each transfected cell line was shown to be independent of IL-3 for growth. Furthermore clones of each cell line that had the same doubling time as empty vector-transfected cells (MSCV Ba/F3) cultured in IL-3 were chosen (data not shown). Expression of the PTKs led to differential effects on protein tyrosine phosphorylation (Fig. 1*B*).

Comparative Analysis of the Effect of Expression of Leukemogenic Tyrosine Kinases on the Transcriptome—GeneChip microarrays have been used extensively in the study of oncogene effects on the transcriptome. Exon arrays now allow a genome-wide, exon-level analysis on a single array to investigate alternative splicing and gene expression.

RNA was produced from each cell line, and the effects of the oncogene on the transcriptome were assessed. The exon microarray data indicated that there were relatively few common changes (data are available upon request) at the gene level imposed on the Ba/F3 cells by the six PTKs. Where up-regulation occurred, 86 probe sets (matching 44 genes) showed changed levels (>2-fold) in six of six oncogenes with a further 311 probe sets (194 genes) in five of six and 949 probe sets (598 genes) in four of six. Given the extensive nature of these arrays (*i.e.* the large number of probe sets they contain), calculated false discovery rates (the proportion of probe sets that would appear in each category by chance) are 75.4, 55.5, and 48.1% respectively. Clustering exons to genes enables higher confidence of an effect. However, only five genes (comprising 33 probe sets) showed up-regulation of at least half of their exons in six of six oncogenes (Table I).

Analysis of the down-regulated genes showed a similar pattern. Although down-regulation appeared to be more prevalent than up-regulation, still only 396 probe sets (from 117 genes) were significantly altered by six of six oncogenes with 1232 (391 genes) and 2742 (1110 genes) being down-regulated by five of six and four of six oncogenes, respectively. Again the nature of these experiments means a calculated false discovery rate for these probe sets of 17.5, 22.2, and 22.7%, respectively. Further analysis at the gene level showed that only 12 genes (comprising 122 probe sets) have 2-fold (or greater) down-regulation of at least half of their exons in six of six oncogenes (Table I).

Although a more in-depth analysis is in progress to detect variation in gene splicing, it remains clear from these data that the mode(s) of action of the six oncogenes studied has very few common features at the transcriptome level. The lack of commonality at the transcriptome level does not exclude the possibility that these oncogenes do in fact share a common pathway for transformation potentially via splicing. Further analysis

TABLE I
Genes identified as changing in all six oncogenes on analysis of the exon array data
To be included the genes must show change greater than 2-fold in over half of the probe sets.

ID	Gene description	Probe sets changing (total number of exonic probe sets)
Genes up-regulated in all 6 cell lines (greater than 2-fold)		
ENSMUSG00000023367	Hepatocellular carcinoma-associated antigen 112	5 (8)
ENSMUSG00000029810	*LR8* protein	8 (10)
ENSMUSG00000031773	cDNA sequence AK220210	17 (28)
ENSMUSG00000026399	CD55 antigen	7 (10)
ENSMUSG00000057596	Expressed sequence AI451617	4 (6)
Genes down-regulated in all 6 cell lines (greater than 2-fold)		
ENSMUSG00000018924	Arachidonate 15-lipoxygenase	14 (15)
ENSMUSG00000034652	CD300A antigen	11 (11)
ENSMUSG00000019122	Chemokine (CC motif) ligand 9	8 (9)
ENSMUSG00000030483	Cytochrome P450 2b13	6 (10)
ENSMUSG00000032089	Interleukin 10 receptor, α	13 (11)[a]
ENSMUSG00000029446	Phosphoserine phosphatase	7 (9)
ENSMUSG00000021108	Protein kinase C, η	16 (17)
ENSMUSG00000027073	Proteoglycan 2	5 (7)
ENSMUSG00000054366	RIKEN cDNA A330102H22	5 (10)
ENSMUSG00000040302	RIKEN cDNA C030048B08	6 (10)
ENSMUSG00000010342	*TEX14*	21 (37)
ENSMUSG00000040828	*TMEM146*	10 (13)

[a] Denotes where probe sets associated with this gene locus but not mapped to an exon were also changed by >2-fold.

of this data set is under way to identify splicing effects of PTKs.

We then considered other means for PTKs to elicit common effects. It is now well established that oncogenic tyrosine kinases not only post-translationally modify proteins but also initiate post-translational regulation of the proteome via processes such as altered rates of translation or stability via post-translational modification, *e.g.* ubiquitination (22). Only analysis of proteomic changes can reveal these targets because mRNA levels will remain unperturbed. We have shown previously that oncogene-induced changes in the proteome do not always correlate to changes in the transcriptome (12). In addition changes seen in protein levels in primitive hematopoietic cells undergoing development do not correlate precisely to changes in the transcriptome (11). We therefore investigated the effects of these oncogenes on the proteome.

Isobaric Tagging for Relative Quantification from Eight Samples in a Single Mass Spectrometry Experiment—We have shown previously that the iTRAQ isobaric tagging approach gives highly reproducible data in the sense that peptides from the same protein show similar relative quantification patterns, and also biological replicates show markedly similar trends (11, 12). A new generation of iTRAQ isobaric tagging reagent was used for eight-channel peptide relative quantification proteomics. This uses the same chemistry to label peptides via free amine groups as described previously (13) but is constructed to enable eight different samples to be relatively quantified in a single tandem mass spectrometry experiment (Fig. 2, *A* and *B*). In the eightplex reagent, reporter ion masses are now 113.1–119.1 Da and 121.1 Da. The mass at 120.1 is omitted to avoid contamination from phenylalanine immonium ion (*m/z* 120.08). The extra channels are added by increasing the overall mass of the balance group. In addition, all mass differences are now encoded using ^{13}C and ^{15}N around a ring structure in the reporter group (Fig. 2*A*). A representative MS/MS spectrum showing the new reporter ion region is shown in Fig. 2*B*.

To ensure appropriate relative quantification using the new reagent, a defined protein mixture was digested with trypsin and labeled with the eight-channel iTRAQ reagent (Fig. 2*C*) as described previously with the four-channel iTRAQ reagent (12). The appropriate protein relative quantification levels were found to be achieved with the approach used with the mixture of proteins (see legend to Fig. 2). Analysis of a standard eight-protein mixture showed that in all cases this method is capable of protein relative quantification (all proteins except ovotransferrin and carbonic anhydrase were present in a 1:1 ratio in the isobarically tagged tryptic digest samples; see Fig. 2 legend for detail). It should be noted that, in our hands, the approach slightly underestimated the degree of -fold change anticipated, shown by the *diamond symbols* (Fig. 2*C*), a feature we have observed previously with the fourplex reagent. These data show that the eightplex iTRAQ can be used for assessment of relative PTK effects on the proteome.

Comparative Analysis of the Effect of Expression of Leukemogenic Tyrosine Kinases on the Proteome—To analyze potential common proteome differences elicited by the six PTKs, eight-channel isobaric tagging with tandem mass spectrometry was used. Proteomics data was obtained from two ex-

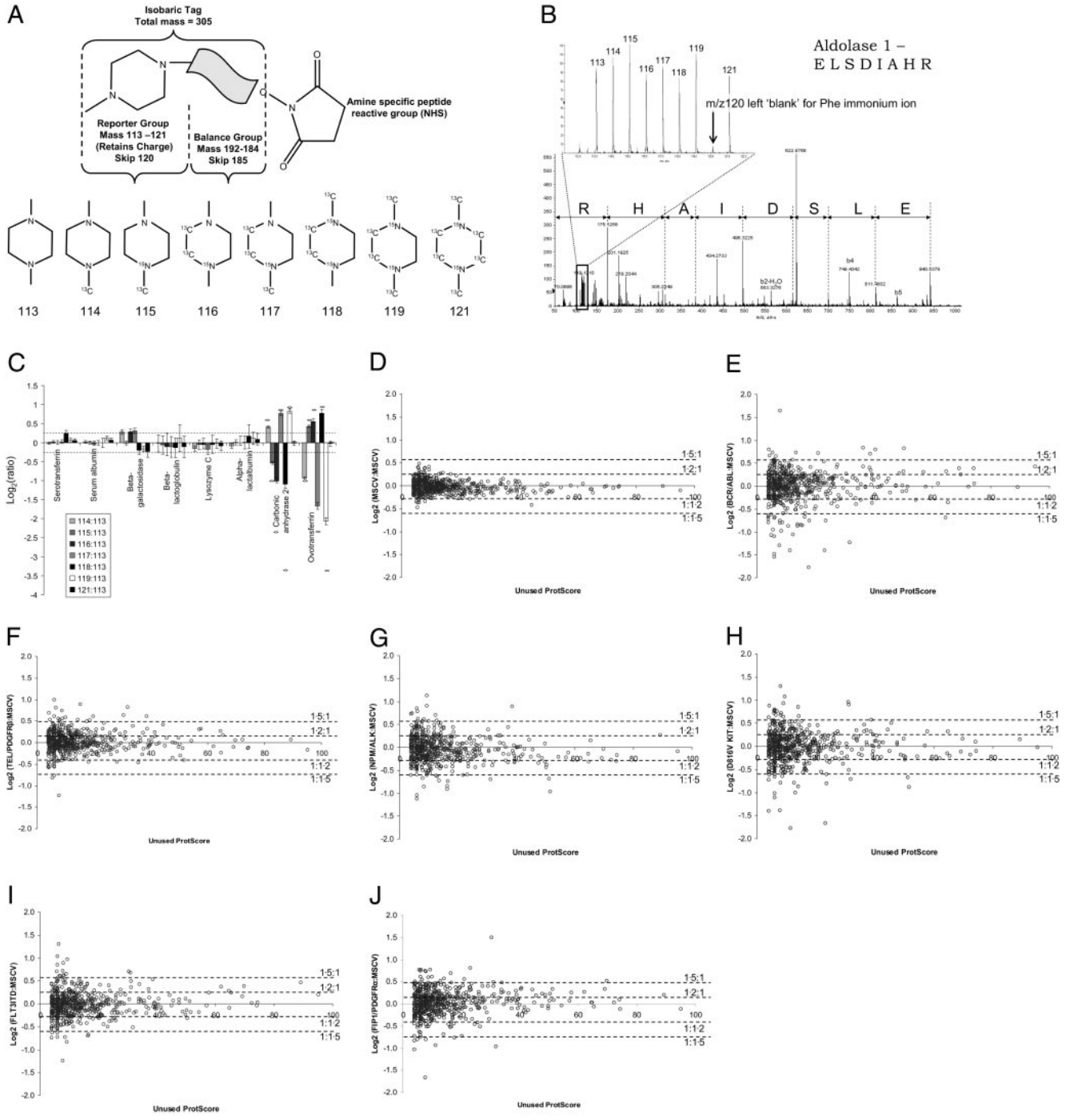

FIG. 2. **Analysis of protein expression in six oncogene-transfected cell lines by eightplex isobaric tagging.** *A*, a schematic of the new eight-channel (eightplex) iTRAQ reagent, including the isotope coding in the new reporter groups. Reporter masses are sequential, but omit 120 Da as this is also the mass of a phenylalanine immonium ion. *B*, an example of the data generated is shown with the sequence determined by the pattern of fragment ions (*lower panel*) and quantification derived for the intensities of the eight reporter ions (*upper panel*; sample relative abundance, 1:1:1:1:1:1:1:1). *C*, relative quantification of a mixture of eight proteins mixed in predetermined quantities prior to isobaric tagging. The relative ratios in which the proteins were mixed is shown on the figure using *diamond symbols*. The *bars* shown represent 95% confidence interval levels. *D*, further validation of the method using biological sample replicates is shown where the ratios of the MSCV:MSCV Ba/F3 control cells (mixed 1:1 ratio) were labeled and compared for protein ratios assessed using the iTRAQ approach. The *x axis* indicates the protein identification score (unused score from ProteinPilot software; see "Experimental Procedures") that represents the sum of the $\log_{10}$(percentage of confidence of peptide identification) of each unique peptide associated with only that protein. Each spot represents a protein, and only proteins with a score of >4 are included. *E–J* show the MSCV Ba/F3 cell isobaric tag relative quantification plotted against oncogene PTK-transfected Ba/F3 cells.

periments where 1380 protein and 1614 protein identifications with a confidence score greater than 95% were made, respectively. Searching against a reversed database allowed calculation of false discovery rates for these two experiments as 0.146 and 0.806% at the protein level, respectively. Relative quantitation analysis was performed on these data sets separately and then averaged together to give a list of 1886 proteins (see supplemental Table 1).

In each case, labeling efficiency was estimated to be >98% by comparing the total number of identified potential reactive sites (*i.e.* N termini and lysine side chains) with the number of iTRAQ modifications at these sites. In addition, there was some concern that although the total amount of protein analyzed in the eightplex experiment was the same as in a standard fourplex (*i.e.* 400 μg) there is now only half of the material in each channel (50 μg per eightplex compared with 100 μg per fourplex). However, we compared the number of peptides identified with confidence >90% in this data set and a fourplex data set from similar samples (*i.e.* whole cell lysates) and showed that in each case around 8% of these peptides were not included in the final data set due to low iTRAQ reporter ion intensity. Therefore there appears to be no deleterious effects of halving the amount of sample in each channel to allow expansion from a fourplex to an eightplex relative quantification experiment.

Further analysis was performed to select proteins where there was a high confidence of protein identification and relative quantification using criteria defined in the analysis software (see "Experimental Procedures" and legend to supplemental Table 2). Relative quantification of the effects of six PTK oncogenes on 829 proteins is therefore shown where two channels were biological replicates of mock-transfected Ba/F3 cells included to confirm reproducibility of the approach as was seen in our previous work (11, 12). Fig. 2*D* shows that the ratio for the majority of proteins lies between 0.8 and 1.2, the window described previously (12) beyond which a protein can be considered to be potentially changed in expression level. For comparison the relative protein expression levels of oncogene-transfected Ba/F3 cells compared with MSCV Ba/F3 are shown (Fig. 2, *E–J*). These demonstrate the quantitative variability in the effect of each oncogene on the proteome as a whole with BCR/ABL having the largest gross effect.

The changes noted for the oncogenes in both experimental replicates were: BCR/ABL: 168 proteins changed, 77 down-regulated, 91 up-regulated; TEL/PDGFRβ: 67 proteins changed, 22 down-regulated, 45 up-regulated; NPM/ALK: 87 proteins changed, 59 down-regulated, 28 up-regulated; D816V KIT: 106 proteins changed, 54 down-regulated, 52 up-regulated; FLT3ITD: 73 proteins changed, 38 down-regulated, 35 up-regulated; and FIP1/PDGFRα: 124 proteins changed, 43 down-regulated, 81 up-regulated. The identities of these proteins are shown in supplemental Tables 3–8.

Protein changes were examined further using a dendrogram approach to identify any clustering of oncogene effects. The analysis showed neither clustering of type 3 leukemogenic receptor tyrosine kinases (FLT3ITD, D816V KIT, FIP1/PDGFRα, and TEL/PDGFRβ) nor oncogenes associated with myeloproliferative disorders (Fig. 3*A*). D816V KIT did cluster with FLT3ITD, but this was in a group of three with NPM/ALK, the only PTK in the set associated with lymphoma. Construction of an Edwards-Venn diagram containing proteins with a change in expression between control cells and each oncogene-transfected cell population also revealed the lack of major overlap in effect between any of the oncogenes (Fig. 3*B*). Surprisingly FIP1/PDGFRα and TEL/PDGFRβ oncogenes showed very little similarity. We therefore conclude that leu-

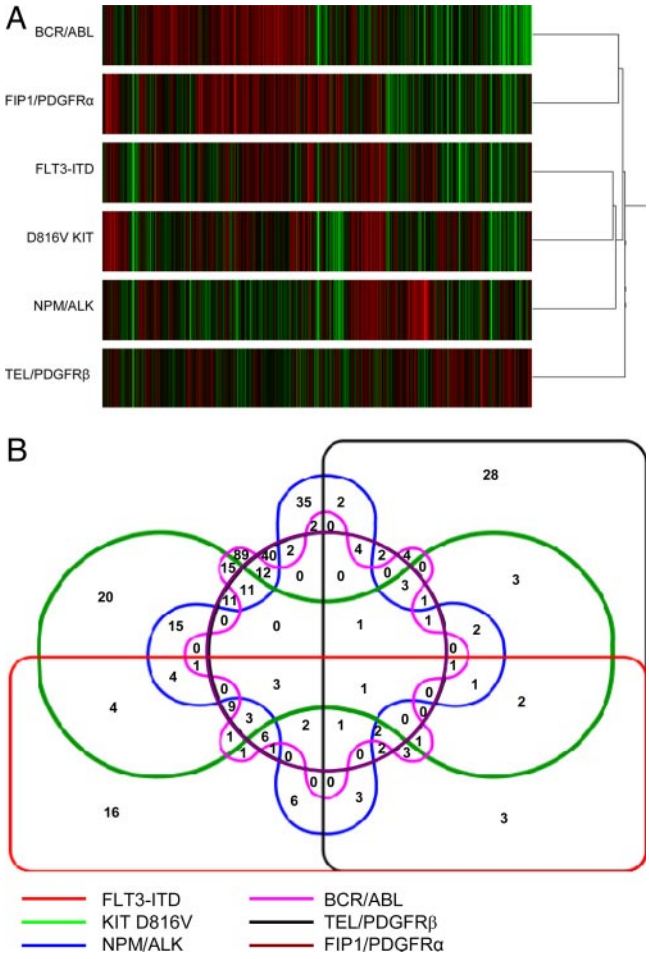

FIG. 3. **Analysis of potential oncogene relationships.** *A*, dendrogram analysis was performed using the condition tree option in the Genespring GX microarray analysis package (Agilent Technologies). Oncogenes are clustered according to similar protein expression profiles observed in iTRAQ ratios. *Green* indicates a decrease, *red* indicates an increase, and *black* indicates no change in iTRAQ ratios. The dendrogram tree structure illustrates the relationship between the oncogenes. The *varying length* of the *horizontal* "*branch*" indicates the degree of similarity between the oncogenes; a *shorter branch* indicates a greater degree of similarity. *B*, an Edwards-Venn diagram constructed to illustrate the protein changes commonly caused by the oncogenes.

TABLE II

Proteins whose expression is altered by the expression of four or more oncogenes

Proteins shown are those where a confident assessment of a common decrease or increase in four or more of the six oncogenes was found. To be included the data must contain more than two peptides and have a -fold change (control *versus* oncogene) <0.8 or >1.2 with a *p* value of less than 0.05. The significant changes are shown in bold; the underlined values represent "changes" where the confidence levels were not met.

Accession number	Identity	-Fold change (oncogene compared with control)						Number of oncogenes in which change were seen
		BCR/ABL	TEL/PDGFRβ	NPM/ALK	KIT D816V	FLT3ITD	FIP1/PDGFRα	
trm|Q9R126	Eosinophil-associated ribonuclease 7	**0.12**	**0.38**	**0.06**	**0.04**	**0.17**	**0.32**	6
spt|P20152	Vimentin	**1.42**	**1.63**	**1.90**	**1.20**	**1.43**	1.13	5
spt|Q9EQU5	SET protein (phosphatase 2A inhibitor I2PP2A)	**0.74**	**0.68**	**0.77**	0.96	**0.80**	**0.85**	5
trm|Q99LB4	Capping protein (CAPG)	**0.51**	0.91	**0.74**	**0.51**	**0.67**	**0.61**	5
trm|Q69Z71	Scinderin	**0.26**	1.48	**0.60**	**0.39**	**0.41**	**0.25**	5
spt|P25444	40 S ribosomal protein S2 (S4)	**1.52**	1.00	1.02	**1.21**	**1.24**	**1.29**	4
spt|P62843	40 S ribosomal protein S15 (RIG protein)	**1.36**	0.97	0.98	**1.21**	**1.18**	**1.27**	4
spt|P47968	Ribose-5-phosphate isomerase	**0.56**	1.07	**0.71**	**0.68**	**0.58**	0.93	4
trm|Q69ZN0	Guanine deaminase	**0.46**	0.79	**1.27**	**0.54**	**0.55**	1.31	4
spt|Q61878	Eosinophil granule major basic protein precursor	**0.15**	0.47	0.47	**0.21**	**0.32**	**0.23**	4
spt|O35381	Acidic nuclear phosphoprotein pp32	**0.75**	0.95	**0.61**	0.88	**0.79**	**0.75**	4
cra|mCP22728.2	Ferritin light chain 1	**0.48**	0.83	**0.39**	0.44	**0.44**	**0.53**	4
trm|Q8CJ71	Heterogeneous nuclear ribonucleoprotein	**0.73**	0.97	**0.79**	**0.70**	**0.80**	0.90	4
trm|Q9D154	Serine protease inhibitor EIA	**0.34**	1.34	**0.65**	**0.31**	**0.71**	2.03	4

kemogenic tyrosine kinases have pleiotropic effects on global protein expression. This is underscored by analysis of the gene ontogeny for proteins affected (in expression level) by the oncogenic PTKs in that effects on many protein groups were recorded, and the effects of each oncogene were broadly similar (data not shown).

The number of proteins within the data set affected by >4 of the oncogenic PTKs was small (Table II) and covers a wide variety of protein functions. Eosinophil associated ribonuclease 7 is a member of the RNase A superfamily involved in inflammatory processes mediated by eosinophils. CAPG binds the end of actin filaments and regulates cellular structure (23). Scinderin (adseverin) cleaves actin filaments and has been shown previously to be changed in expression in the hematopoietic stem cell to progenitor cell transition (24). Vimentin is a member of the intermediate filament family of proteins that is increased in expression with all PTKs except FIP1/PDGFRα. Two related protein phosphatase inhibitors (SET proteins) were decreased in four of six and five of six PTK-transfected cell lines, respectively; D816V KIT cells were the only cells to show no change in either. These proteins have been implicated in BCR/ABL-mediated transformation (25, 26). The two 40 S ribosomal proteins S2 and S15 both displayed an increase except with the TEL/PDGFRβ- and NPM/ALK-transfected Ba/F3 cells. This relative lack of common effects demonstrates diversity in mechanism of action for leukemogenic PTKs.

Correlation of Peptide Expression Data to Exon Expression Data—In the past, comparison of protein expression data with transcript expression has been achieved, but it is always undermined by the inability of both standard expression array platforms and bottom-up proteomics analyses to accurately resolve different isoforms. We have established novel bioinformatics approaches to link these proteomics data to the exon array data to check the correlation between protein/peptide abundance and mRNA expression. This enables analysis of oncogenic PTK effects on splice isoform generation. We can now assess the correlation of proteome to transcriptome at three levels: peptide to exon probe sets; >1 peptide to exon data; and protein expression (summated peptide data) *versus* gene-specific expression (summed exon data). Pearson correlation between exon arrays and peptide data, at the exon *versus* peptide level, for MSCV *versus* oncogene was 0.47 for BCR/ABL, 0.22 for NPM/ALK, 0.28 for TEL/PDGFRβ, 0.30 for FLT3ITD, 0.25 for D816V KIT, and 0.40 for FIP1/PDGFRα. The data set will be the subject of a much more in-depth analysis, but the main conclusion relevant here is that there is relatively poor correlation between changes at the exon and peptide levels. Thus a majority of protein changes cannot be predicted by mRNA analysis. Selected examples of this correlation and its anomalies are illustrated in Fig. 4 where protein expression data for proteins shown in Table II have been compared with exon array data. The transcriptome and proteome expression levels for ribose-5-phosphate isomerase and eosinophil major basic protein show a high degree of correlation. The data for CAPG, however, clearly demonstrate that the data obtained from RNA expression levels do not always correlate to protein expression levels. The fidelity of

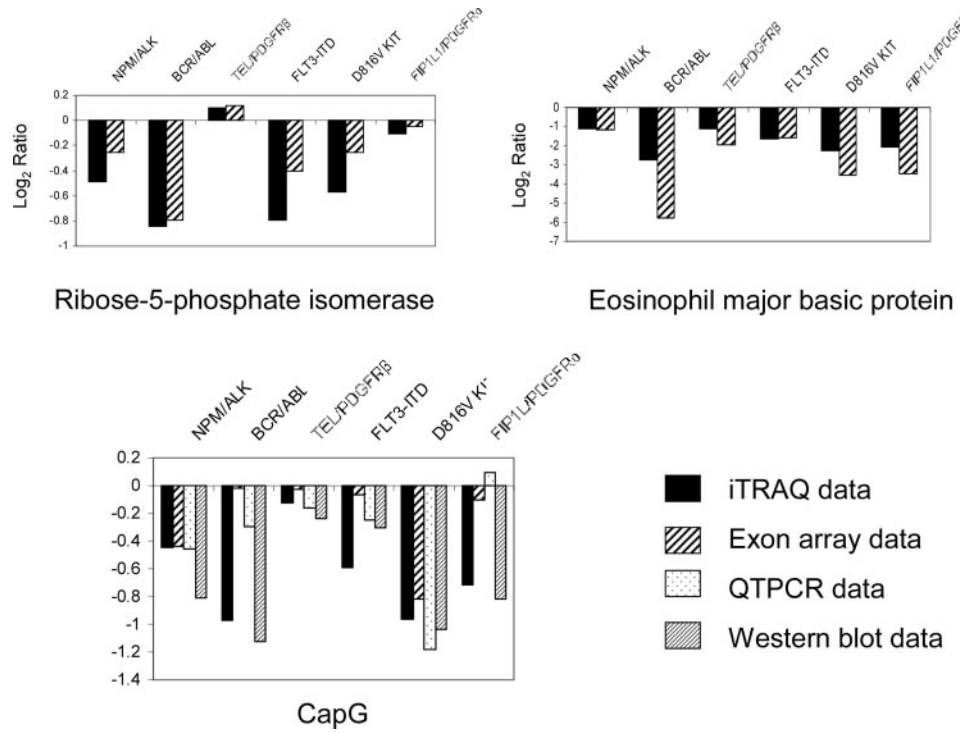

FIG. 4. **Correlation of iTRAQ and exon array data.** Representative examples of proteins found to change in greater than four of six oncogenes are shown. Exon array, iTRAQ, Western blot, and QRT-PCR data are displayed as a -fold change compared with MSCV control.

the exon array and iTRAQ data was confirmed by use of QRT-PCR and Western blot analysis. The data for CAPG also illustrate that different oncogenes can affect a protein/pathway by different mechanisms. Although CAPG was downregulated in both NPM/ALK- and BCR/ABL-transfected cells this occurred via different mechanisms: NPM/ALK cells showed a decrease in transcript level resulting in a similar decrease in protein level; BCR/ABL cells displayed no decrease in transcript, yet protein levels are decreased.

Correlation of Proteomic Changes to Motility Effects—Systematic proteomics analysis enables oncogenic effects on specific functional pathways to be assessed and further analysis to be performed. The oncogene-transfected cell lines were shown to display differential cell motility in serum-free conditions (Fig. 5). In cells expressing BCR/ABL motility was decreased as observed in previous studies (27, 28). However, expression of TEL/PDGRFβ in these conditions enhanced motile response as did D816V KIT. Vimentin, scinderin, and CAPG are proteins associated with the cytoskeleton and motility that were affected by almost all of the six PTKs in this analysis. Thus their modulation by oncogenic PTKs can be seen to be inconsistent with the differential effects observed on motile response.

We therefore further analyzed the data for all proteins affected by each PTK whose gene ontology suggests association with motility. Within this set were proteins that were perturbed (increased or decreased) in expression levels by BCR/ABL and perturbed in the opposite direction by TEL/PDGFRβ and D816V KIT. Two candidates were found that could affect motile responses. These were high mobility group protein B2 (HMGB2) and Septin 1. HMGB2 has been associated previously with altered motility and is therefore a candidate target protein for altered motile responses instigated by these oncogenes.

DISCUSSION

The discovery of PTKs that are associated with leukemogenic transformation has led to investigation of the specific roles for these kinases in transformation (29–31). Often a singular oncogenic PTK is the subject of a focused study seeking to identify downstream effectors modulated by that specific kinase, such as motility effects or genomic stability mediated by BCR/ABL (32, 33). The molecular components of any oncogene-mediated change are then targets for analysis in respect of the effects of other PTKs: much information exists describing the effects of BCR/ABL on a variety of pathways and processes (34, 35), but there is much less substantial scientific literature on the effects of other oncogenic kinases on these pathways. In this fashion the determination of common and uncommon events promoted by specific kinases will progress at a relatively slow rate. Given the growing number of kinases associated with the leukemias we sought to establish whether novel systems biology approaches can determine the effects of PTKs on the proteome and transcriptome.

To achieve a meaningful foundation data set the require-

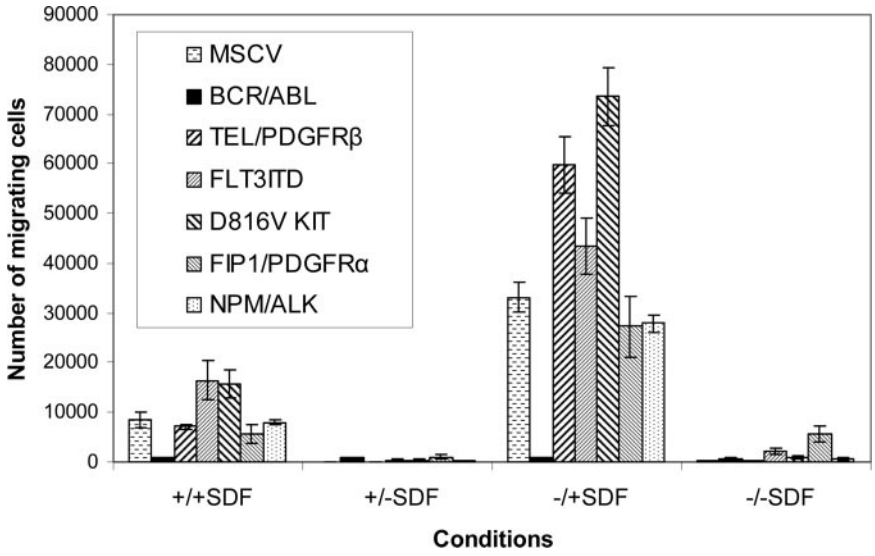

FIG. 5. **The effect of oncogenic PTK expression on the chemotactic response to SDF-1.** The SDF-1-induced chemotactic response of Ba/F3 cells transfected with the oncogenes shown was assessed in Boyden chamber assays. 10^5 cells were added to the top well, and 100 ng/ml SDF-1 was added to either the top well, bottom well, or both wells. The number of cells in the bottom well was counted after 6 h. Results are the mean ± S.E. of three experiments.

ment is for a common cellular background on which to examine the effects of PTKs. Ba/F3 cells are commonly used in studies on leukemogenesis and are therefore suitable for initial analyses (36). Although this approach demands follow-up studies on other cell lines and primary material, a successful primary screening strategy on a common background will inevitably save time in understanding common events seen in transformation.

The analysis of the data set we constructed showed very few common leukemogenic PTK-mediated events. At the exon/transcript level, these include increases in hepatocellular carcinoma-associated antigen, LR8 protein, and CD55 antigen. CD55, an inhibitor of the complement system, is widely expressed in malignant tumors and reported to have a role in decreasing complement-mediated tumor cell lysis, cell motility, and metastasis (for a review, see Ref. 37). Other probe sets also showed changes in six of six oncogenes, but these were accompanied by no changes in other exons from that gene. This may represent alternative splicing events. However, given the large number of probe sets on each array (over 1.4 million probe sets) it is almost inevitable that many false positives will be called "changed" and that a subset of these will be called in all oncogenes by random chance. The false discovery rates for identification of an up-regulation in six of six oncogenes suggests that 75.4% of those identified could be expected by chance. We reasoned that, because this analysis was done at the probe set level, those probe sets that were falsely called changed in six of six oncogenes would be unlikely to map to the same gene locus by random chance. So in this background only genes that had a large proportion of its probe sets shown to be altered in all oncogenes can be reported as potential common targets. Unfortunately the protein products of these genes were not identified in the proteomics analysis presumably due to their low abundance. Data generated using exon arrays offer the opportunity to consider the possible effects of transformation by individual oncogenes on splice variants and gene expression. Further analysis on exon-specific effects to consider oncogene-mediated splice variant generation is under way.

It is now becoming more accepted that changes in the proteome do not necessarily occur as a result of changes in transcription. To look for common oncogenic targets in the proteome, we utilized the newly developed eight-channel iTRAQ reagent to simultaneously assess protein expression in cells expressing six different oncogenes with the remaining two channels taken up by biological replicates of empty vector-transfected cells. The eight-channel iTRAQ system is a redesigned version of the fourplex reagent to allow incorporation of the extra isotopes required to extend the reporter ion mass range. This requires a new "balance" group and minor modifications to the reporter while retaining the same labeling chemistry via an *N*-hydroxysuccinimide ester. In our hands, this new reagent retained many of the features of the fourplex reagent in that labeling efficiency remained high, we saw an apparent increase in signal in a UV detector (at 214 nm) during SCX fractionation, and peptides appeared to have slightly increased retention on the SCX column. There is no evidence that peptide elution is different for peptides labeled with different iTRAQ reagents (on either SCX or reverse phase medium); peptide fragmentation is good, and the iTRAQ reporter ion signal(s) are generally among the most abundant peaks in the majority of spectra. Using a defined mixture, we showed that, like the fourplex, the eightplex was capable of detecting differences in peptide levels between two samples, but the degree of change was underestimated. This is unimportant in a study where a low false positive rate is required as in our studies. We have therefore demonstrated that eight-channel relative quantification mass spectrometry can compare protein profiles (and in the future phosphopeptide profiles (38) in hematopoietic cells).

Once again, however, the proteomics analysis supported the hypothesis that the leukemogenic properties of the six

oncogenes shared little in common. Only one protein was shown to be affected by all six oncogenes, namely eosinophil-associated ribonuclease 7 (also known as ribonuclease 7). Other proteins found to be altered in five of six or four of six oncogenes include two members of the SET protein phosphatase family (I2PP2A and acidic nuclear phosphoprotein pp32). I2PP2A is known to be involved in BCR/ABL-positive chronic myeloid leukemias (25). Also several proteins identified are related to the actin cytoskeleton structure. This would suggest a common effect on cell shape or cell motility. Functional assays for motility suggest that, although BCR/ABL appears to decrease the motile response to SDF1 and both TEL/PDGRFβ and D816V KIT increase motility, this pattern does not correlate with the change in expression of these proteins. Likewise further analysis of the data for proteins whose expression follows this pattern yields two candidates, Septin 1 and HMGB2. These are both therefore candidates for proteins that play a role in oncogene-mediated alterations in motility. However, it is clear that, because other motility-associated proteins remain unaltered or do not follow this pattern, the oncogenes that do affect motility probably do so using distinct mechanisms.

The exon array approach has been coupled to proteome analysis to match relative quantification at the peptide and exon probe set level. This analysis is potentially more powerful than a gene *versus* protein analysis due to the methods of data generation. Standard proteomics and microarray technologies each may sample different parts of a protein transcript, so it could be that a comparison is actually between probe sets at the beginning of a transcript *versus* peptides from the end of the protein. This may be the reason why proteome and transcriptome changes correlate poorly in previous studies. Using exon arrays allows a much higher resolution mapping, matching peptide expression to the levels of the mRNA from the exon that codes for it. However, this analysis also shows a general lack of correlation between changes in expression at the transcript and proteome levels. Interestingly the correlations between protein and gene were better than those between peptide and exon (data not shown) presumably because averaging peptides/exons reduces the effect of outlying data points. To confirm these data for a case where there was no correlation, CAPG, the proteome and transcriptome data, respectively, were checked using Western blotting and relative quantitative PCR. This confirmed the accuracy of the systematic analyses and also revealed further differences between the actions of the oncogenes. Where both NPM/ALK and D816V KIT down-regulated CAPG by decreasing the rate of mRNA transcription, BCR/ABL, FLT3ITD, and FIP1/PDGFRα all showed no change in *CAPG* mRNA levels and therefore down-regulate the protein either by a slower rate of translation or by increased degradation. These findings promote the idea that proteome analysis (at both the protein and peptide/phosphopeptide levels) should be a key element of systematic analyses of oncogene effects.

Here we have shown that simple extension of effects seen with one PTK to other leukemogenic oncogene tyrosine kinases is inappropriate. There is little correlation between six PTKs on the proteome seen using the approach we validated here. Rather than deflecting the search for commonalities the study suggests a focus on specific organelles (to identify common effects on lower abundance proteins) and enrichment of post-translationally modified proteins as a target for further analyses. The present analysis showed no correlative links between type III receptor tyrosine kinases, few common effects ellicited by the six PTKs and a raft of effects across many gene ontology groups. Identification of a group of proteins commonly affected enables development of ideas on common events required in PTK action that contribute to transformation.

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