Identification of Novel MAP Kinase Pathway Signaling Targets by Functional Proteomics and Mass Spectrometry

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Summary

Functional proteomics provides a powerful method for monitoring global molecular responses following activation of signal transduction pathways, reporting altered protein posttranslational modification and expression. Here we combine functional proteomics with selective activation and inhibition of MKK1/2, in order to identify cellular targets regulated by the MKK/ERK cascade. Twenty-five targets of this signaling pathway were identified, of which only five were previously characterized as MKK/ERK effectors. The remaining targets suggest novel roles for this signaling cascade in cellular processes of nuclear transport, nucleosome excision repair, nucleosome assembly, membrane trafficking, and cytoskeletal regulation. This study represents an application of functional proteomics toward identifying regulated targets of a discrete signal transduction pathway and demonstrates the utility of this discovery-based strategy in elucidating novel MAP kinase pathway effectors.

Introduction

The mitogen-activated protein kinase (MAPK) pathway, consisting of the MAP kinase kinases (MKKs) 1 and 2 and extracellular signal-regulated kinases (ERks) 1 and 2, has been implicated in diverse cellular processes, including proliferation, transformation, and cell differentiation (Marshall, 1995; Lewis et al., 1998). Activation of this signaling cascade elicits variable responses in different cell types, presumably due to cell-specific downstream targets. Traditional approaches using single readout assays are insufficient to comprehensively describe signaling targets unique to distinct cell and tissue types. Thus, systematic approaches are needed that report global responses to signaling pathways, such as the MAPK pathway, in the context of cell-specific protein expression.

New approaches to address complex biological systems include the use of cDNA microarrays and oligonucleotide chips to monitor changes in mRNA expression (Fodor et al., 1993; Schena, et al., 1995). Applications to signaling include studies on the serum response of S. cerevisiae, gene arrays have been used to identify novel transcriptional targets of Fus3 and Kss1 MAPK signaling pathways (Madhani et al., 1999; Roberts et al., 2000). However, mRNA-based assays are unable to detect covalent modification, regulated translation, or proteolysis, which are key regulatory events in signal transduction mechanisms. Furthermore, studies in human liver and S. cerevisiae have shown that mRNA levels correlate poorly with corresponding protein levels (Anderson and Seilhamer, 1997; Gygi et al., 1999). In S. cerevisiae, quantitation of 106 gene products showed a linear correlation coefficient < 0.4 between mRNA and protein levels, after excluding the 11 most abundant proteins (Gygi et al., 1999).

Proteomics provides a complementary and potentially more comprehensive approach to the analysis of signaling mechanisms by resolving the expressed proteins of the cell ("proteome") followed by protein sequencing and identification (Williams and Hochstrasser, 1997). Changes in protein profiles during signaling events can be monitored using two-dimensional (2D) gel electrophoresis (O’Farrell, 1975; Gorg et al., 1988) or related techniques, detecting alterations in expression levels, posttranslational modification, proteolytic processing, or alternative message splicing. Furthermore, identification of proteins in subpicomolar quantities via mass spectrometry is routine, and posttranslational modifications can be detected and mapped (Wilm et al., 1996). Thus, in the same experiment, targets of a signaling pathway can be identified by changes in transcriptional and posttranscriptional regulation, providing novel mechanistic insight into how signaling events elicit complex biological responses.

Applications of functional proteomics to signaling problems include studies of molecular responses to osmotic stress, PDGF stimulation, and phosphatase function (Blomberg, 1997; Alms et al., 1998; Soskic et al., 1999). In this study, we present an application of functional proteomics toward identifying targets of a specific signal transduction pathway. We and others have previously demonstrated that activation of the MKK/ERK pathway by phorbol ester or cytokine treatment of human erythroblasts in megakaryoblastic cell lines is necessary and sufficient for the induction of differentiation into megakaryoblastic cell lines (Melemed et al., 1997; Rouyez et al., 1997; Whalen et al., 1997). Within this biological context, we sought to identify cellular targets regulated by the MAPK pathway, using a functional proteomic strategy involving pathway activation by expression of active kinase mutants and pathway repression by a cell-permeable inhibitor.

This analysis identified 41 distinct protein targets of phorbol ester signaling, distinguishing targets regulated by posttranslational modification from those regulated by synthesis or degradation. Of these, 25 were identified as downstream targets of the MAPK pathway, of which only 5 represent previously characterized MAPK path-
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Identification of MAPK Pathway Targets
A strategy involving selective activation and inhibition of the MAPK pathway was used to discriminate MAPK pathway targets from among the PMA-responsive protein changes. In order to activate the MAPK pathway, K562 cells were transiently cotransfected with constitutively active (CA) mutants of MKK1 and MKK2 and harvested 24 hr posttransfection, using wild-type (WT) MKK1 and MKK2 as controls. Parallel transfections using a GFP reporter demonstrated >95% transfection efficiency under these conditions (data not shown). Activation of ERK2 above basal levels only occurred in cells expressing CA-MKK1/2, although WT-MKK expression was equivalent to CA-MKK (Figure 3B). 2D gel analysis revealed that 65 of the 91 spot changes responsive to PMA were reproduced by direct activation of the MAPK pathway with CA-MKK1/2 (Figure 3A).

U0126, a cell-permeable inhibitor of MKK1/2 (Favata et al., 1998), blocks megakaryocytic differentiation in K562 cells by inhibiting the ability of MKK1/2 to activate ERK1 and ERK2 following PMA stimulation (Figure 2B). U0126 inhibited 60 of the observed protein changes induced by PMA concomitantly with 95% inhibition of ERK2 activity, implicating these proteins as targets of the MAPK pathway (Figures 2A and 4). Of these, 35 were completely blocked (e.g., Figure 2A, U-5, U-42, U-24 and U-25, U-1 and U-2), and 26 were partially blocked after 240 min (e.g., Figure 2A, U-40), reflecting differential thresholds in molecular responses to MKK/ERK. Treatment of cells with U0126 for 240 min in absence of PMA showed only four changes, corresponding to decreased intensities of U7, U42, U86, and U87. All of these were previously characterized as direct or indirect targets of MAP kinase (see below), indicating that the only observed effect of U0126 was to repress basal MKK activity. Thus, in our experiments, U0126 exhibited specificity for the MKK/ERK pathway.

All PMA-induced protein changes that were inhibited by U0126 were also induced by direct activation of the pathway through CA-MKK1/2 (e.g., Figure 2A vs Figure 3A). However, five PMA-dependent changes were induced by CA-MKK1/2 transfection for which no significant U0126 effect was observed (U-26, U-27, U-29, U-31, and U-36). Of these, U-26 through U-31 represent posttranslationally modified forms of cytokeratin-8 (Table 1), a substrate of both MAPK and protein kinase C (PKC) (Omary et al., 1992; Ku and Omary, 1997). Thus, incomplete inhibition of PMA-induced posttranslational modifications by U0126 would be expected for this protein. Complex behavior was observed for U-36 (WASP interactor protein), which was downregulated by PMA in a U0126-independent manner, but upregulated upon CA-MKK1/2 expression. Other protein changes occurred upon transfection of CA-MKK1/2, which may reflect targets of sustained MAPK signaling not observed after short term stimulation with PMA (data not shown). Otherwise, good agreement was observed between the PMA responses that were induced with active MKK and those repressed with U0126. This allowed us to classify 66% of the PMA-dependent protein changes as targets of the MAPK pathway (Figure 4).

Novel Targets of the MAPK Pathway
Peptides were extracted from each spot by in-gel tryptic digestion, and protein identities were determined by...
Figure 1. Detection of PMA-Responsive Proteins in K562 Cells

Whole-cell extracts (200 μg) of nontreated proliferating K562 cells (A) or cells treated for 240 min with 10 nM PMA (B) were separated by 2D PAGE, and protein changes (indicated by red arrows) were detected using gel analysis software as described in Experimental Procedures. Arrows show proteins that (A) decrease in intensity following PMA treatment or (B) appear de novo or increase in intensity over the time course of treatment. Experiments were also run using extracts of cells treated for 20, 60, and 120 min and 24 hr (data not shown).
Figure 2. Differential Kinetics and Sensitivity to Inhibition by U0126

(A) Selected regions of 2D gels illustrate proteins U-5, U-42, U-24 and U-25, U-40, and U-1 and U-2 (indicated by arrows) comparing K562 cells that were untreated (0 min) or treated with 10 nM PMA for 20 or 240 min (left panels). Cells were also pretreated with 20 μM U0126 prior to addition of PMA and incubation for 240 min (right panels).
proteins that were human homologs of known mouse or rat entries. Names of identified proteins, their NCBI sequence identification numbers (gi), spot designation, and cluster numbers are listed in Table 1. Information about sequence coverage, peptide sequence, and observed versus experimental molecular mass is provided as supplemental data (www.molecule.org/cgi/content/full/6/2/1343/DC1).

Twenty-five of the distinct proteins were found as MAPK pathway targets, based on criteria of inhibition by U0126 and activation by CA-MKK (Table 1), referred to as MKK-regulated proteins (MRPs). Surprisingly, only five were previously known to be regulated by this pathway. These included MKK2; stathmin and cytokeratin 8, which are known substrates of ERK (Marklund et al., 1993; Ku and Omary, 1997); eukaryotic translation initiation factor 4E (eIF-4E), a substrate of the ERK-activated kinase, MNK1 (Waskiewicz et al., 1999); and myeloid cell leukemia sequence 1 (MCL-1). Thus, the assay elucidated several expected targets of the MAPK pathway. Other expected targets, such as ERK2, Raf-1, and PKC, have pI values outside the range examined in this study and were not observed.

The remaining twenty targets of the MKK/ERK pathway revealed in this analysis included proteins involved in signal transduction, nuclear transport, DNA repair and chromatin assembly, mRNA processing, membrane trafficking, cytoskeletal regulation, proteolysis, and protein folding. Two MKK/ERK targets, small glutamine-rich tetra-tricopeptide repeat (TPR)-containing protein (SGT) and MRP-4, have recently been cloned; therefore, little Figure 3. Proteomic Changes Responsive to Constitutively Active MKK1 and MKK2 Mutants
(A) Selected 2D gel regions illustrate proteins in Figure 2 from cells transiently cotransfected with WT-MKK1 + WT-MKK2 (left panels) or CA-MKK1 + CA-MKK2 (right panels) harvested 24 hr after transfection.
(B) Western blots of whole-cell extracts (10 µg/lane) harvested 12 or 24 hr posttransfection, probed with anti-HA antibody (upper panel) to detect MKK expression or anti-phosphoERK1/2 (lower panel) to detect ERK1/2 activation. WT-MKK1 migrates faster than WT-MKK2, whereas CA-MKK1 and CA-MKK2 comigrate.

peptide mass fingerprinting, searching the NCBI non-redundant database. Protein identification was confirmed by mass spectrometric sequencing (MS/MS), matching sequence tags against the nonredundant and Unigene EST databases. In many examples, multiple spots represented the same protein, revealing their relationship by posttranslational modification or variation due to alternative splicing (Figure 4). Thus, the 91 protein changes together represent 41 distinct proteins. In all cases, the observed pI and molecular weights of identified proteins were consistent with calculated values or variations reported in the literature. Peptide mass and sequence tag information identified 85% of the unknowns, including 32 proteins that matched human entries exactly and 3 proteins that were human homologs of known mouse or rat entries. Names of identified proteins, their NCBI sequence identification numbers (gi), spot designation, and cluster numbers are listed in Table 1. Information about sequence coverage, peptide sequence, and observed versus experimental molecular mass is provided as supplemental data (www.molecule.org/cgi/content/full/6/2/1343/DC1).

In 17 cases, unmodified forms as well as modified forms of PMA or MKK/ERK targets were detected (Figure 5). Time courses for some of these targets behaved as expected from conversion of precursors to products, showing little or no change in total protein expression (Figures 6A–6F, 6I, and 6J). Others showed net gain or loss of total protein at 24 hr, consistent with concomitant synthesis or degradation (Figures 6G, 6H, 6K, and 6L). For the most part, modification of MKK/ERK targets occurred rapidly, correlating with the kinetics of MKK2 (Figures 6A–6G). Only two examples showed rates of modification slower than MKK, corresponding to SGT (Figure 6G, U66, cluster 19) and MRP-2 (Figure 6H, U24

(B) Western blot of whole-cell extracts (3 µg/lane), probed with antibody recognizing diphosphorylated ERK2, the predominant isoform in K562 cells. Cells were pretreated with 0.2% (v/v) DMSO carrier or U0126 prior to addition of 0.1% (v/v) ethanol or PMA for 240 min.
(C) Cluster analysis of PMA time courses allowed segregation of 89 protein spot changes into 20 distinct patterns. Points indicate average values and standard deviations of normalized intensities of “n” proteins within each cluster, calculated as described in Experimental Procedures. For convenience, values in each cluster are scaled to the highest value which is set equal to 100. See Table 1 for the cluster number of each unknown.
Western blots of PMA-stimulated K562 cells showed dose- and time-dependent proteolytic processing, from its full-length form with apparent mass of 58 kDa to a stable 38 kDa product (Figure 7A). Processing was complete 4 hr after stimulation and was blocked by U0126. At 24 hr, the full-length form reappeared in a manner inhibited by cycloheximide. Identical behavior was observed in response to CA-MKK1/2 expression or treatment of other cell lines with PMA (HeLa-S3, HeLa-COL, and 293 HEK), and processing was blocked by the proteasome inhibitor, MG132 (data not shown). Therefore, MKK/ERK functions to rapidly convert HR23B to a proteolytic processed form, and recovery of the full-length form requires new protein synthesis. Treatment of HeLa-S3 cells with UV also induced processing to the 38 kDa fragment, although less pronounced in this cell line (Figure 7B). These results validate HR23B as a novel target of MKK/ERK signaling under the physiological condition of UV irradiation.

HR23B from cells metabolically labeled with 32P-orthophosphate showed elevated phosphorylation of the 38 kDa fragment in response to PMA treatment of K562 cells (Figure 7C) and UVC treatment of HeLa cells, in a manner inhibited by U0126 (Figure 7D). We found no evidence for phosphorylation of full-length HR23B, although posttranslational modification of this form would be expected based on 2D gel information. We hypothesize that the full-length form may be phosphorylated and rapidly proteolyzed such that incorporation of radiolabel is too low to observe, or alternatively, it may be modified by a different chemistry followed by proteolysis and phosphorylation. Although we do not know the function of the 38 kDa processed form of HR23B, both mammalian HR23B and yeast Rad23 associate with 26S proteasome complexes, and a homolog, HR23A, is proteolyzed in a cell cycle-dependent manner (Schauber et al., 1999; Kumar et al., 1999). Our results are consistent with a model in which MKK/ERK signaling promotes covalent modification and proteasome-catalyzed processing of HR23B.

Discussion

This study represents a large-scale investigation of a specific signaling pathway by proteomics and demonstrates the feasibility of identifying pathway effectors on a global level by using a combination of activators and inhibitors to respectively isolate the “on” and “off” states of pathways. Overall, 35 of the 41 distinct proteins responsive to phorbol esters were matched successfully to sequences of known gene products. Thus, 85% of the proteins were identifiable using information from the available human genome sequence. Twenty-five proteins were found to be regulated by MKK/ERK, based on criteria of suppression by the U0126 inhibitor and activation by CA-MKK. The remaining 16 proteins were regulated by other pathways controlled by phorbol ester, including conventional and atypical PKCs. Indeed, coronin-2 and PTP1B members of the latter set are known as direct substrates for PKC (Flint et al., 1993; Parente et al., 1999).

The redundancy of proteins related by posttranslational modification and alternative splicing was esti-
Table 1. Summary of Identified Proteins and Functional Roles

<table>
<thead>
<tr>
<th>Functional Category</th>
<th>MKK Regulated Proteins</th>
<th>Phorbl Ester Responsive Proteins</th>
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<tbody>
<tr>
<td>Nuclear transport</td>
<td>RAN-binding protein 1* [938026: U90(2)]</td>
<td>Importin-alpha-1 subunit* [4504895: U59(8)]</td>
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<tr>
<td>Apoptosis</td>
<td>Myeloid cell leukemia sequence 1 [4235637 U71(20)]</td>
<td>Protein tyrosine phosphatase 1B [938026: U90(2)]</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>Retinaldehyde dehydrogenase 2 [3970842: U74(15)]</td>
<td>Translin associated protein X [1770576: U41(9)]</td>
</tr>
<tr>
<td>DNA repair, nucleosome assembly, and translocation</td>
<td>MAP kinase kinase 2* [547915: U103(3), U104(8)]</td>
<td></td>
</tr>
<tr>
<td>DNA repair, nucleosome assembly, and translocation</td>
<td>UV excision repair protein RAD 23 homolog A** [1709983: U52(~), U53(14), U93(2)]</td>
<td></td>
</tr>
<tr>
<td>DNA repair, nucleosome assembly, and translocation</td>
<td>UV excision repair protein RAD 23 homolog B** [1709985: U55(10), U56(10), U95(4)]</td>
<td></td>
</tr>
<tr>
<td>DNA repair, nucleosome assembly, and translocation</td>
<td>Nucleosome assembly protein-2 (NAP1L4)* [5174613: U11(18), U20(10)]</td>
<td></td>
</tr>
<tr>
<td>Golgi stacking, vesicle trafficking, and membrane proteins</td>
<td>Replication protein-A2* [4506585: U91(1)]</td>
<td>Coat assembly complex (\beta) adaptin subunit***</td>
</tr>
<tr>
<td>Proteolysis and protein folding</td>
<td>Human Golgi reassembly stacking protein of 55 kDa [9911866: U94(2)]</td>
<td>WASP interactor protein (sorting nexin 9 family) [1400621 U36(5)]</td>
</tr>
<tr>
<td>mRNA processing and translation</td>
<td>110 KD cell membrane glycoprotein [2949400: U61(10)]</td>
<td>Rab27a* [1710023: U68a(11)]</td>
</tr>
<tr>
<td>mRNA processing and translation</td>
<td>Proteasome zeta chain* [88168: U3(14)]</td>
<td>Chaperonin containing T-complex polypeptide (\beta)-subunit [2559012: U42(9)]</td>
</tr>
<tr>
<td>mRNA processing and translation</td>
<td>Eukaryotic translation initiation factor 4E*** [1352435: U40(3), U69(10)]</td>
<td>Eukaryotic translation initiation factor 4A-I***** [4503529: U92(3)]</td>
</tr>
<tr>
<td>mRNA processing and translation</td>
<td>Heterogeneous nuclear ribonucleoprotein C1/C2*** [133261: U7(10), U101(5), U103(5)]</td>
<td>Seryl-tRNA synthetase* [1351173: U43(12)]</td>
</tr>
<tr>
<td>mRNA processing and translation</td>
<td>Heterogeneous nuclear ribonucleoprotein K*** [241478: U13(3), U14(3), U15(7), U16(~), U17(3), U18(1), U19(~), U20(7), U21(14), U22(10), U23(8), U35(14), U75(13), U78(14), U80(8), U85(9), U97(3)]</td>
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</tr>
<tr>
<td>Cytoskeletal regulation</td>
<td>APC-binding protein EB1* [2135057: U67(9)]</td>
<td>Coronin-2* [4895039: U38(1), U76(7)]</td>
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<tr>
<td>Cytoskeletal regulation</td>
<td>Statmin*** [134973: U86(10), U87(10), U88(4)]</td>
<td>Cytokeratin-18*** [125083: U8(7), U81(6)]</td>
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<td>Cytoskeletal regulation</td>
<td>L-Plastin*** [134673: U10(4), U11(7), U12(12)]</td>
<td>Cytokeratin-8*** [2506774: U26(18), U27(17), U28(17), U29(17), U30(17), U31(11), U32(11)]</td>
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<tr>
<td>Unknown function</td>
<td>Nucleosome assembly protein-2 (NAP1L4)* [2498400: U61(10)]</td>
<td>Human homolog of mouse RP42* [5174613: U1(18), U2(10), U3(11), U8(1)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>Replication protein-A2* [4506585: U91(1)]</td>
<td>Acetyl serotonin N-methyl transferase-like* [4757794: U77(13), U99(1)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>Small glutamine-rich TPR-containing protein* [2909372: U51(9), U63(9), U64(9), U65(8), U66(19)]</td>
<td>Human homolog of mouse RP42* [6166507: U68b(11)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>MRP-4-4 &quot;hypothetical protein&quot; [4653540: U46(13), U47(13), U48(13)]</td>
<td>PRP-2 &quot;unknown gene product&quot; [3252826: U9(8)]</td>
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<tr>
<td>Unknown function</td>
<td>MRP-2* [296006: U10(4), U11(7), U12(12)]</td>
<td>PRP-1* [4100621 U36(5)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>MRP-2* [1647573: U24(16), U25(15)]</td>
<td>PRP-3* [1400621 U36(5)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>MRP-3 [U45(13)]</td>
<td>PRP-4 [1400621 U36(5)]</td>
</tr>
<tr>
<td>Unknown function</td>
<td>MRP-4 [U54(14)]</td>
<td></td>
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</table>

Identified proteins are specified by: [NCBI sequence ID: unknown number (cluster number)]. Further information about sequence coverage, peptide sequences, and observed versus expected MW and pI can be viewed at http://www.molecule.org/cgi/content/full/6/2/1343/DC1.

* Posttranslational modification detected by 2-D gel electrophoresis upon PMA treatment and/or expression of CA-MKK 1/2.

** Spot U-68 is a mixture of Rab27a and the human homolog of mouse RP42 proteins.

\* Less than 2-fold mRNA change observed by Affymetrix in PMA-stimulated HL60 cells (Tamayo et al., 1999).

\* Protein behavior inconsistent with mRNA change observed by Affymetrix (Tamayo et al., 1999), DNA microarray in serum-stimulated fibroblasts (Iyer et al., 1999), or cDNA subtraction in Ras-transformed fibroblasts (Zuber et al., 2000).

\* Protein behavior consistent with mRNA change observed by Affymetrix (Tamayo et al., 1999).

\*\* Posttranslational modification of at least half of the targets was unambiguous, based on the identification of precursor and product forms. Kinetic analysis further showed that 95% of the proteins responded within the first 20 min of PMA treatment, faster than most transcriptional events (Figure 2C). We therefore believe that the majority of targets identified in this study are regulated through mechanisms involving posttranslational modification, even in cases when precursor or product forms of these targets were not detected. For example, detection would be obscured if net changes in precursor forms were less than 1.5-fold or modified products were rapidly degraded. Only two targets, MCL-1 (cluster 20) and RALDH2 (cluster 15), showed delayed responses expected from transcriptional induction. Both are known
targets for transcriptional induction in response to signaling (Corcoran and Maden, 1999; Townsend et al., 1999). However, not all slow responses were explainable by mechanisms involving protein synthesis. MRP-2 showed an unexplained delay in the initial kinetics of conversion between two forms clearly related by post-translational modification (Figure 6H), which likely reflects complex regulation, for example, involving alterations in cellular compartmentalization of ERK.

Published mRNA expression monitoring studies include a time course of HL60 cell responses to PMA measured by Affymetrix, a time course of fibroblast responses to serum measured by DNA microarrays, and a study of Ras-dependent transformation targets in fibroblasts measured by cDNA subtraction (Iyer et al., 1999; Tamayo et al., 1999; Zuber et al., 2000). We found that 19 of the 35 proteins identified in our study were represented in at least one of the data sets (Table 1). All of these showed directionality of changes that were opposite to or kinetics that were inconsistent with the protein behavior. Only 4 of 15 matches with the Affymetrix data set showed 2-fold changes in mRNA expression following PMA treatment of HL60 cells. Three showed directionality of mRNA changes that were opposite to the effects observed in our study. Only RP-A2, which declined by 4 hr after PMA treatment in mRNA levels in the Affymetrix study, changed in a manner consistent with the behavior of protein in our study, which decreased by 20 min and remained low for 24 hr. In summary, only one out of 19 targets showed mRNA changes consistent with the proteomic results, supporting our prediction that most are regulated by posttranslational modification rather than transcriptional induction or repression.

Our results reveal many previously unidentified targets of the MAPK pathway, indicating that its regulatory functions are broader than previously realized. Among these are hHR23A and B and replication protein A2, which are implicated in global genome nucleotide excision repair (de Laat et al., 1999). hHR23A and B are each rapidly modified posttranslationally in response to MAPK signaling, suggesting a novel role for this pathway in mechanisms that sense DNA damage. Our analysis indicates a role for MAPK in creating a stable processed form of HR23B, which is also observed in response to UV (Figure 7). In addition, a potential role for MAPK in chromatin assembly is suggested by rapid modification of nucleosome assembly protein 2 (NAP-2/NAP1L4), a homolog of the NAP-1 nucleosome assembly complex subunit, which recruits nucleosomes to naked DNA templates in vitro and appears to function in chromatin assembly (Rodriguez et al., 1997). The identification of the human homolog of GRASP55, which functions in the stacking of Golgi cisternae (Shorter et al., 1999), is of interest in light of a recent study suggesting a requirement for MKK1 in Golgi reorganization during mitosis (Acharya et al., 1998). Finally, modulation of Ran-BP1 may reflect a role for MAP kinase in nuclear transport.

Figure 5. Mass Spectrometric Analysis of an Uncloned Protein Responsive to MKK/ERK
(A) MALDI-TOF peptide mass spectrum of the tryptic digest of U-24. Peptide mass data alone were insufficient to establish identity of this protein, requiring MS/MS sequencing analysis.
(B) Zoom scan of the 688.9 Da parent ion. Resolution of the isotopic peaks of this peptide identified it as the doubly charged ion of MH+ = 1376.60 Da detected by MALDI-TOF (indicated by brackets in [A]).
(C) MS/MS spectrum of the 688.9 Da ion, showing b ions and y ions labeled in the peptide sequence and spectrum. Dashed symbols indicate fragment ions with intensity >3-fold above background. Mass/charge ratios of observed b3±9 and b11 ions were 256.1, 384.2, 830.5, 993.5, 1121.6, and 1192.6 Da. Based on MS/MS sequencing, U-24 matched six entries in the human dbEST database, all of which represented the same uncloned gene, referred to as MRP-2.
MAPK Pathway Targets Identified by Proteomics

Figure 6. Comparison of Kinetic Behavior of Precursors and Products of MKK/ERK- and PMA-Responsive Targets

Shown are 12 of 16 instances in which proteins corresponding to both precursors and products of posttranslational modification were identified. Targets were modified by the MAPK pathway (A–H) or PMA (I–L). Values indicate percentage of volume intensities, as described in Experimental Procedures.

Several proteins identified in this study may be involved in promoting cellular differentiation. First, MCL-1, a Bcl-2 family member, is upregulated in response to MKK/ERK and is likely to be involved in maintaining cell survival during differentiation (Kozopas et al., 1993). Second, induction of retinaldehyde dehydrogenase 2 (RALDH2), which catalyzes the final step in retinoic acid biosynthesis, suggests secondary signaling through retinoic acid, which induces differentiation in many blood cell types. Nerve growth factor (NGF), an activator of the MAPK pathway, upregulates transcription of RALDH2 in adult dorsal root ganglia and induces neurite outgrowth by the production of retinoic acid (Corcoran and Maden, 1999). Third, control of translation is suggested by MKK/ERK regulation of hnRNP-C and -K. During megakaryocytic differentiation of K562 cells, hnRNP-C binds the 5′-UTR and relieves translational inhibition of PDGF B chain mRNA (Sella et al., 1999). Similarly, hnRNP-K translationally silences erythroid 15-lipoxygenase (LOX) mRNA, a key erythroid marker, by binding to its 3′ UTR, preventing 80S ribosome assembly (Ostareck et al., 1997). Taken together, these findings suggest that the MAPK pathway induces megakaryocyte markers and suppresses erythroid markers through translational regulatory mechanisms involving hnRNP phosphorylation and modulation of RNA binding affinity. Finally, potential links between morphological changes during K562 differentiation and cytoskeletal regulators targeted by MAPK include the actin binding protein, L-plastin, and the tubulin binding protein, stathmin.

Many of the MAPK targets represent novel or previously uncharacterized genes. SGT and MRP-4 have been fully cloned but as yet have no experimentally determined functions. SGT was identified as a cellular binding partner of parvovirus protein NS1 and contains three TPR domains (Cziepluch et al., 1998), suggesting that it may be a scaffolding protein responsive to MKK/ERK signaling. Four targets (MRP1, 2, 3, and 5) did not match with fully cloned genes in current databases, although MRP-2 matched several entries representing the same gene in the human dbEST database. Sequence analysis failed to reveal significant homology of MRP-2 and MRP-4 to known genes that would have predicted function. Thus, the proteomic strategy enables identification of completely novel targets of MAPK signaling.

In summary, proteomics combined with mass spectrometry is a powerful method for analyzing signal transduction pathways, with the ability to detect global changes in signaling events, as well as distinguish changes due to posttranslational modifications from those due to expression and degradation. In particular, early events that occur within minutes can be monitored in a manner complementary to high throughput assays for mRNA expression which detect later events under transcriptional control. Our study illustrates the enormous potential of discovery-based protein methodologies for identifying new signaling targets and for giving insight into mechanisms for cellular control.
Experimental Procedures

Cell Culture Experiments and Transient Transfection
K562 cells were cultured as described (Whalen et al., 1997). Prior to PMA addition, $6 \times 10^6$ cells/10 ml were plated into fresh media in 10 cm dishes and incubated for 6 hr at 37°C. Time courses were initiated with 10 nM PMA, and cells were harvested after 20, 60, 120, and 240 min by centrifugation (1000 rpm, 5 min, 4°C), washed twice with ice cold phosphate-buffered saline containing 1 mM EDTA and EGTA, frozen in N2(l), and stored at -80°C. Control cells were treated with ethanol carrier and harvested immediately.

In experiments examining MAPK pathway targets, K562 cells were pretreated with 20 μM U0126 (Promega) for 30 min prior to PMA addition. Alternatively, cells were transfected by electroporation as described (Whalen et al., 1997), coexpressing human MKK1(WT) or MKK2(WT) or constitutively active mutant MKK1(R4F) or MKK2(KW71A) or MKK2(N3/S218E/S222D/S226D). Twelve hours posttransfection, cells were centrifuged and either harvested immediately or replated in fresh media and harvested after another 12 hr.

Extract Preparation and 2D Electrophoresis
Lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% IPG buffer pH 4–7) was applied to the strips followed by constant 3500 V, with focusing complete after 80,000 Vh. Prior to the second dimension, strips were incubated (15 min, 28.5°C) in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris [pH 8.8], 30% glycerol), first with 65 mM DTT (reductive) and second with 243 mM iodoacetamide (alkylating). Equilibrated strips were inserted onto sucrose gradient SDS–PAGE gels (20 × 22 cm; 8.5%–17.5%), sealed with 0.5% agarose, and 2D gels were run at 12°C (BioRad Protean IIxi) overnight for 2600 Vh. Analytical gels were silver stained as described (Blum et al., 1987), while preparative gels were silver stained using a low fixation recipe (Shevchenko et al., 1996). Gels were preserved by air drying between sheets of moistened cellophane.

Western Blotting and Immunoprecipitation
Cell extracts were resolved by SDS–PAGE and transferred to PVDF membrane (NEN). Membranes were reacted 1 hr with primary antibody followed by goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories, 1:7500 dilution) and visualized by enhanced chemiluminescence (Amersham). Expressed MKK1/2 were detected using 12CA5 mouse anti-HA antibody (1:5000), and hHR23B was detected using mouse clone 16 antibody (Transduction Laboratories, 1:250).

K562 or HeLa-S3 cells (1–2 × 10⁶ cells/dish) were washed twice with phosphate-free RPMI-10% dialyzed FCS, incubated in this media for 2 hr, and treated with 1 μCi 32P-orthophosphate for 6 hr. Cells were pretreated with 20 μM U0126 or DMSO carrier prior to PMA treatment. Alternatively, media was removed, cells were washed with PBS, irradiated with UVC (254 nm, 0–300 J/m²) or UVB (300 nm, 3.9 W, 60 s), and the same media was returned to the cells. Cells were incubated for 60 min at 37°C followed by one PBS wash and harvesting. Extracts were centrifuged at 10,800 rpm for 10 min at 4°C, and HR23B was immunoprecipitated from the clarified supernatants with clone 16 antibody. Immune complexes were bound to protein G-Sepharose, separated by SDS–PAGE, and transferred to PVDF membrane, which was first exposed to film by autoradiography, then probed by Western blotting with anti-HR23B.
2D Gel Quantification and Analysis

2D gels were scanned, and digital images were analyzed for changes in protein patterns using the image analysis software Melanie 2.1 (Appel et al., 1997). Each protein change was assigned a temporary identity (U-1 through U-104). Silver-stained proteins were quantified using Melanie 3.0. Intensities were measured as percentage of volume, corresponding to pixel intensities integrated over the area of each spot and divided by the sum over all spots in the gel. The intensity of each spot was then corrected for subtracting a background intensity of equal area. Cluster analysis was performed by direct visual inspection, sorting patterns of temporal changes in intensity between 0 and 24 hr. For protein spots within each cluster, intensities at each time point were normalized to the average intensity over all time points. Average values and standard deviations of the normalized intensities for all spots at each time point were then calculated and plotted versus time. Proteins U-16, U-19, and U-52, which were altered within 20 min, were not included in the cluster analysis due to difficulties in quantifying these spots.

Preparation of Samples for Mass Spectrometry

Preparative 2D gels of nontreated and PMA (240 min) treated K562 cell extracts were silver stained using the low fixation protocol, and unknown proteins were excised directly from gels. Since each spot differed in protein quantity, the number of gels required for each unknown varied from 1 to 4 for high abundance proteins to 15–18 for lower abundance proteins, accumulating ~500 fmol of each protein. Excised protein spots were destained (Gharahdaghi et al., 1999), and in-gel digestions were performed according to Shevchenko et al. (1996), except that reduction and alkylation steps were omitted because cysteines were carboxyamidomethylated during gel preparation, and modified porcine trypsin (Promega) was employed (100–200 ng/digestion). If necessary, recovered peptides were desalted and concentrated using C18 ZipTips (Millipore), eluting peptides in 50% (v/v) acetonitrile:water.

Mass Spectrometry and Protein Identification

Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager-DE STR, Perkin Elmer) was used to obtain peptide mass information for each unknown sample. Samples were cocryocrystallized with matrix (ω-cyano-4-hydroxy-trans-cinnamic acid, Hewlett Packard) on gold-coated sample plates. Data were summed over 150 acquisitions in delayed extraction mode, with sensitivity ~10 fmol (20 kV accelerating voltage, 10 V guide wire voltage, 100 ns delay). Unknown proteins were identified by peptide mass fingerprinting using ProFound (http://protol.rockefeller.edu/cgi-bin/ProFound), searching against all eukaryotic entries in the National Center for Biotechnology Information (NCBI) nonredundant protein database. On average, 13.6 peptides in each unknown matched peptides expected in the corresponding identified protein. Observed pl and molecular mass information for each unknown was obtained using a Master 2D gel file containing pl and molecular mass information from 40 identified K562 ´landmarkº proteins evenly distributed throughout the gel. Calculated pl and molecular mass data were obtained from EXPASY (http://www.expasy.org/tools/pi_tool.html).

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) provided amino acid sequence data employing a quadrupole ion trap mass spectrometer (LCQ, Finnigan) coupled with a Magic 2002 HPLC pump and stream splitter (Michrom BioResources). Tryptic peptides of high abundance samples were resolved using an acetonitrile gradient over a 320 min inner diameter Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. Cell 97, 727–741.

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