Monitoring of Gene Expression by Functional Proteomics: Response of Human Lung Fibroblast Cells to Stimulation by Endothelin-1[†]

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ABSTRACT: Proteomic methods have been used to monitor changes in protein synthesis in the first 4 h following stimulation of human lung fibroblasts with endothelin-1. Using pulsed [^{35}S]methionine labeling, about 70 proteins with altered protein synthesis could be detected, and the 35 proteins showing the largest changes were identified by mass spectrometry. The observed proteins included unexpected proteins such as Sox5, two isoforms of Rab14, Rab3A, translationally controlled tumor protein, and one protein of previously unknown function. There was a wide range of different kinetic behavior, and groups of functionally linked proteins such as Rab14, nucleophosmin,and cyclin-dependent kinase inhibitor 1B could be detected from similar kinetics. We propose that the functional proteomic methods are competitive with and have some advantages compared to expression profiling methods for monitoring gene expression.

Changes in the complement of cellular proteins as a consequence of regulated changes in gene expression are one of the fundamental characteristics of living cells. The elucidation of these changes in gene expression and the mechanisms by which they are controlled are one of the major undertakings of current biology. At present, changes in gene expression are usually monitored by nucleotide-based methodologies such as expression profiling (*1*), largely because highly efficient methods to amplify the amounts of polynucleotides for subsequent analysis and identification have been developed. However, the nucleotide-based methods have a number of technical and conceptual disadvantages compared to direct analysis of cellular proteins: (A) it is now known that the old paradigm of one gene equals one protein is not correct for eukaryotic cells where splice variants, posttranslational modifications, etc. lead to perhaps six to eight proteins per gene on average (*2*); (B) while 2D electrophoresis methods are commonly used to resolve and display most cellular proteins (*3*), polynucleotides (mRNA) suffer from the problem that analogous multidimensional display methods are not available. This has required the development of 2D arrays of probe molecules to detect different polynucleotides, but this methodology suffers from the need to predefine all possible polynucleotide (protein) sequences, including potential splice variants; (C) the amplification of different mRNAs may not be quantitatively faithful, and the correlation between mRNA levels and cellular protein levels is not high (*4*), both of which make quantitative evaluation of changes in cellular physiology difficult; (D) the nucleotide-based methods are not suitable for following the plethora of posttranslational modifications of proteins which are vital to cellular response and regulation.

Recent advances in methods used for 2D display of proteins and in the use of the high sensitivity of mass spectrometry to identify proteins have led to a resurgence of direct analysis of cellular proteins, which is encompassed in the (re)emerging field of proteomics (*5*, *6*). It has already been shown that the proteomic approach is particularly suitable for deriving information that will be necessary to develop a "new biology" in terms of networks and fluxes as opposed to molecular identities (*6*, *7*). For example, functional proteomic methods have been successfully used for concurrent monitoring of the phosphorylation/dephosphorylation of hundreds of proteins in signaling pathways in cells (*7*, *8*). One of the key requirements of the functional proteomic approach is to extend its application to monitoring protein expression as a consequence of cellular state. In the present paper we present a functional proteomic approach to detection of changes in protein synthesis resulting from stimulation of human lung fibroblast cells with endothelin.

Endothelin is a strong vasoconstrictor with physiological effects on cellular development, differentiation, vasoconstriction, and mitogenesis (*9*, *10*). These multiple physiological effects are mediated via the G-protein-coupled receptors ETA and ETB (*11*−*13*). Endothelin-1 also appears to be a potent mitogen in cultured vascular smooth muscle cells, cardiac myocytes, mesangium, bronchial smooth muscle, and fibroblasts and induces the expression of several protooncogenes (c-fos, c-jun, c-myc) (*14*−*17*).

In the present paper we report a new type of proteomic experiment which uses pulsed [^{35}S]methionine labeling to detect and identify newly synthesized proteins with as little as 1.7 fmol in protein spots on 2D gel electrophoresis plates. For proteins which showed appreciable changes in new protein synthesis between stimulated and unstimulated cells, the identity of the protein was determined by high-sensitivity MALDI[1] mass spectrometry. At present stage of development, these methods have been used to monitor up/down-

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regulation of gene expression at levels of as little as 10 copies of labeled protein/cell.

EXPERIMENTAL PROCEDURES

Materials. Human lung fibroblast CCDLu19 cells and fetal bovine serum (FBS) were from the European Collection of Cell Cultures. Endothelin-1 (ET-1), ammonium persulfate, β-glycerophosphate, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, sodium carbonate, urea, thiourea, Chaps, TEMED, glycine, Trizma bBase, bromophenol blue, Triton X-100, formaldehyde solution, dithiothreitol, and enzymes (antipain, trypsin inhibitor lima bean, trypsin inhibitor soybean, leupeptin) were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Trypsin−EDTA, antibiotic/antimycotic, minimal essential medium (MEM), HBSS, molecular weight marker, nonessential amino acids (NEAA), and L-glutamine were from GibcoBRL (Eggenstain, Germany). Immobiline DryStrips, L-[^{35}S]methionine, and Pharmalyte were from Pharmacia Biotech (Little Chalfont, U.K.). Duracryl (30% acrylamide, 0.8% BIS) was from Genomic Solutions (Huntingdon, U.K.), and complete mini-protease inhibitor cocktail was from Boehringer (Mannheim, Germany). Trypsin was from Promega (Madison, WI). All other chemicals (Fluka and Roth) were of the best grade available.

Cell Culture. CCDLu19 cells were cultured in MEM supplemented with 10% FBS, 2 mM glutamine, 1% NEAA, and 0.8% antibiotic/antimycotic in a water-saturated, 5% CO_2 atmosphere at 37 °C in 75 cm^2 polystyrene Petri dishes.

Pulse Labeling with [^{35}S]Methinonine. Nearly confluent CCDLu19 cells (90%) were washed twice with the pre-warmed labeling medium containing MEM (Eagle) without L-methionine and L-glutamine, 10% FBS, 2 mM glutamine, 1% NEAA, and 0.8% antibiotic/antimycotic, but no [^{35}S]-Met, and incubated in 5 mL of labeling medium for 30 min in a humidified 37 °C, 5% CO_2 incubator. [^{35}S]Met was added to each dish to a final concentration of 30 mCi/mL.

ET-1 Induction. CCDLu19 cells were incubated with or without ET-1 (0.1 mM) for various periods of time at 37 °C. [^{35}S]Met was added to each dish 30 min before the cells were harvested for analysis. After the induction time, the cells were washed twice with ice-cold PBS containing 15 mM sodium pyrophosphate, 100 mM NaF, 100 mM Na_3VO_4, and 2 mg/mL leupeptin, S antitrypsin, L antitrypsin, and antipain. After centrifugation at 1000 rpm for 5 min at 4 °C, the cells were resuspended in 270 μL of IEF sample buffer containing 8 M urea, 2 M thiourea, 4% Chaps, 1% Triton X-100, 65 mM DTT, 10 mM Tris base, 0.8% Ampholyte, and complete miniprotease inhibitor cocktail. The cells were lysed by sonication and centrifuged for 5 min at 4 °C. The supernatant was stored at −80 °C.

2D Electrophoresis. Ready-to-use Immobiline DryStrips (pH 3−10 or 4−7) were reswollen overnight in 250 μL of cell extract. The IEF was carried out up to a total of 70 kVh. Prior to SDS gel electrophoresis, the strips (gels) were incubated in a solution of 20 mg/mL DTT in equilibration buffer for 20 min and subsequently in a solution of 45 mg/mL iodoacetamide in the same buffer for 20 min. SDS−PAGE was performed in 12% polyacrylamide gels (160 mm × 130 mm × 1.5 mm) at 11 °C at a constant current of 40 mA. The second dimension was run until the bromophenol blue front reached the end of the gel. Gels were stained with the Sigma rapid staining kit according to Shevchenko et al. (*18*).

Autoradiography and Image Analysis. X-ray films exposed for different lengths of time and silver- stained gels were scanned on an UMAX Power look III scanner (Umax, Data System, Hsinchu, Taiwan). Image analysis, spot matching, the analysis of changes in protein synthesis, and estimation of pI/M_r coordinates were done using Melanie III software (Bio-Rad, Richmond, CA).

In-Gel Tryptic Protein Digestion. The protein spots were cut out from the SDS−polyacrylamide gel into small pieces of 1 mm^3, washed three times with acetonitrile, dried for 30 min in a speed-vacuum concentrator, and digested according to the modified procedure of Hellmann et al. (*19*).

Mass Spectrometric Analysis. For MALDI mass spectrometry, aliquots of 0.5 μL of the digested solution were applied to a target disk and allowed to air-dry. Subsequently, 0.5 μL of matrix solution (1% w/v α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% v/v trifluoroacetic acid) was applied to the dried sample, and the sample was again allowed to dry. Spectra were obtained using a Bruker Biflex III MALDI-TOF mass spectrometer. For the identification of the protein fragments we used the MASCOT program available at the Matrix Science web site (http://www.matrixscience.com), the MS-Fit program available at the www site at the University of California at San Francisco (http://rafael.ucsf.edu/cgi-bin/msfit), the ProFound program at the www site of Rockefeller University (http://prowl.rockefeller.edu/cgi-bin/ProFound), the PepSearch program at the www site of EMBL in Heidelberg (http://.www.mann.embl-heidelberg.de/Services/PeptideSearch/FR_peptideSearchForm-.html), and TagIdent available on the ExPASy WWW server.

RESULTS

Many thousands of proteins are expressed in typical eukaryotic cells, and this is reflected in the complexity of 2D PAGE patterns typically observed for total protein extracts from such cells (Figure 1B). In contrast, the response of cells to a stimulus may only involve new protein synthesis for a limited subset of the total cellular proteins. It is desirable to have experimental methods that monitor all extant proteins but also emphasize the subset of proteins responsive to the stimulation. In the case of new protein synthesis induced by stimulation of human lung fibroblast cells with endothelin, we have been able to achieve this objective by pulsed, stepwise endothelin stimulation and [^{35}S]methionine labeling of newly synthesized proteins (Figure 1A) followed by detection of radiolabeled proteins. Thus, while the human genome contains at least 35000 proteins (*20*, *21*) and while at least 3000 total proteins are present on the 2D PAGE gels of human lung fibroblasts (Figure 1B), only a much smaller number of proteins showed detectable amounts of new protein synthesis in our experiments (Figure 1C). The number of proteins showing detectable new synthesis clearly depends on the conditions of our experiment, and in this respect it is important to note that in the present experiments we have

[1] Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization.

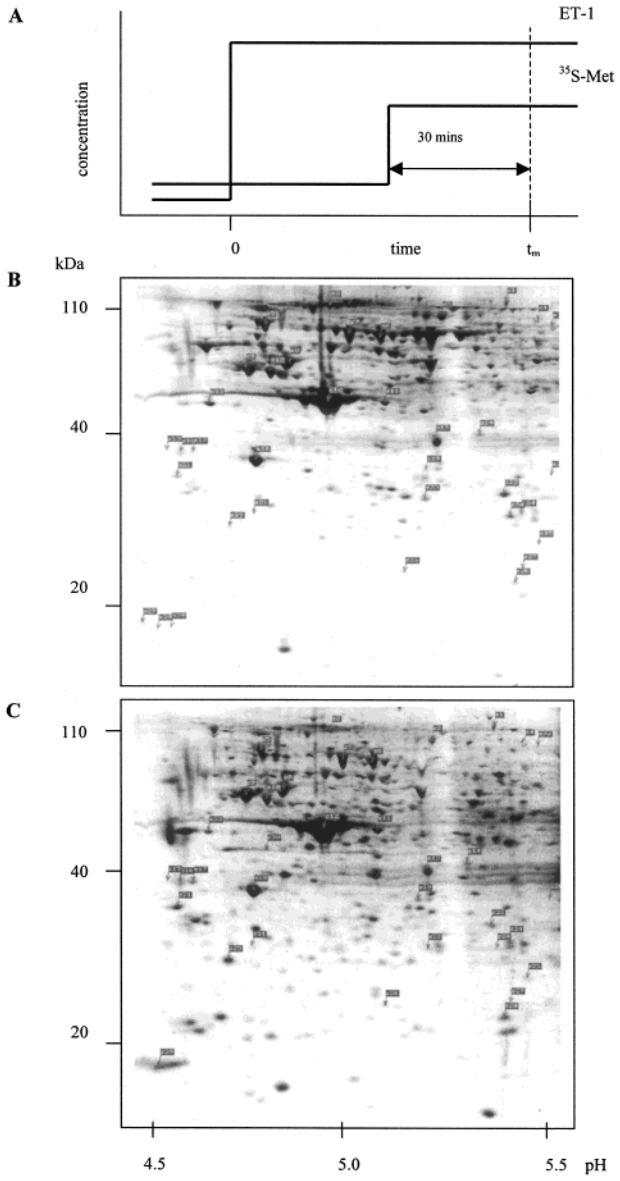

FIGURE 1: 2D gel electrophoresis of proteins from human lung fibroblast cells following stimulation with endothelin-1 and [^{35}S]-methionine labeling between 0 and 30 min. (A) Schematic of the timing used for labeling of newly synthesized proteins. Endothelin stimulation was always started at time zero. For each measurement time, t_m, labeling with [^{35}S]methionine was started 30 min before sampling the cells. (B) Total proteins detected by staining with silver stain. (C) New protein synthesis detected by radioactive labeling with [^{35}S]methionine.

used total amounts of cells and film exposures which should allow the detection of about 10 copies/cell of newly synthesized proteins on the 2D PAGE gels.

To establish which proteins show up/downregulation of synthesis as a function of endothelin stimulation, we have performed [^{35}S]methionine labeling experiments with/without stimulation with endothelin (Figure 2). A total of about 70 proteins showed detectable differences in protein synthesis between stimulated and unstimulated cells. For 35 of these proteins, which showed the largest changes in synthesis, their identity has been determined by mass spectrometry in the present study (Table 1).

There are several important aspects of our experimental scheme, which should be noted. First, the monitoring of radiolabeled proteins is performed at relatively short times after endothelin stimulation is initiated. At present we report stimulation and protein identifications for those proteins for which stimulation could be monitored between 30 and 240 min; i.e., we are primarily analyzing proteins for which changes in protein synthesis are initial/early responses to the endothelin stimulation. Second, because the amount of incorporated radiolabel is measured, new protein synthesis is detected independent of the total amount of a given protein that is present. Third, we have measured rates of new synthesis at distinct times by using a constant, 30 min period of [^{35}S]methionine labeling (Figure 1A). Direct detection of differential changes in protein synthesis, as opposed to changes in total amounts of different cellular proteins, has allowed us to detect cellular responses that would be difficult to identify if changes in total amounts of proteins were measured. Another important observation in these experiments was that although the total amounts of different cellular proteins are known to extend over a dynamic range of concentrations of 10^6 or more, the dynamic range for detectable new protein synthesis over labeling periods of 30 min was much smaller in our experiments and therefore much more easily quantifiable with conventional methods of measuring spot intensities on 2D gels. Finally, it should be noted that detection of 10 copies/cell of newly synthesized protein is more sensitive than the detection of total proteins with general staining methods such as Coomassie blue or silver stain.

For most of these proteins the mass spectrometry identifications were straightforward. In cases where splice variation is known to occur, the specific splice variants shown in Table 1 were observed for vinculin and nucleolar transcription factor. In a few cases, conflicts in the sequence databases were resolved, e.g., the Thr/Ala conflict at position 4 of Ras-related protein RAB-14. In addition, new forms, probably splice variants, of some proteins were observed. For example, we have observed low molecular weight forms of follistatin and Rab14 (Table 1). A particularly interesting case was the observation of two forms of Rab14 (s23 and s28, Table 1) which showed very different new protein synthesis (see below). From the mass spectrometry results, the higher molecular weight form of Rab14 appears to correspond to the complete gene sequence, whereas the lower molecular weight form appears to correspond to a splice variant with deletions at least at the N-terminus. An additional 12 proteins could not be identified in currently available sequence databases. These presumably correspond to presently unknown proteins and/or splice variants and are being further characterized by mass spectrometry.

The changes in new protein synthesis were strongly time dependent, and groups of proteins showing different kinetic behavior could be observed in our experiments (Figure 3). Thus, for example, vilamin, Sox5, and plasminogen activator inhibitor 2 all showed rapid changes in protein synthesis between 30 and 60 min followed by a constant level of upregulation (filamin, Sox5) or downregulation (plasminogen activator inhibitor 2) at later times (Figure 3A). Other groups of proteins showed very different kinetic behavior (Table 1). The Rab14 (long) and Rab14 (short) isoforms also showed very different kinetic behavior (Figure 3D,E).

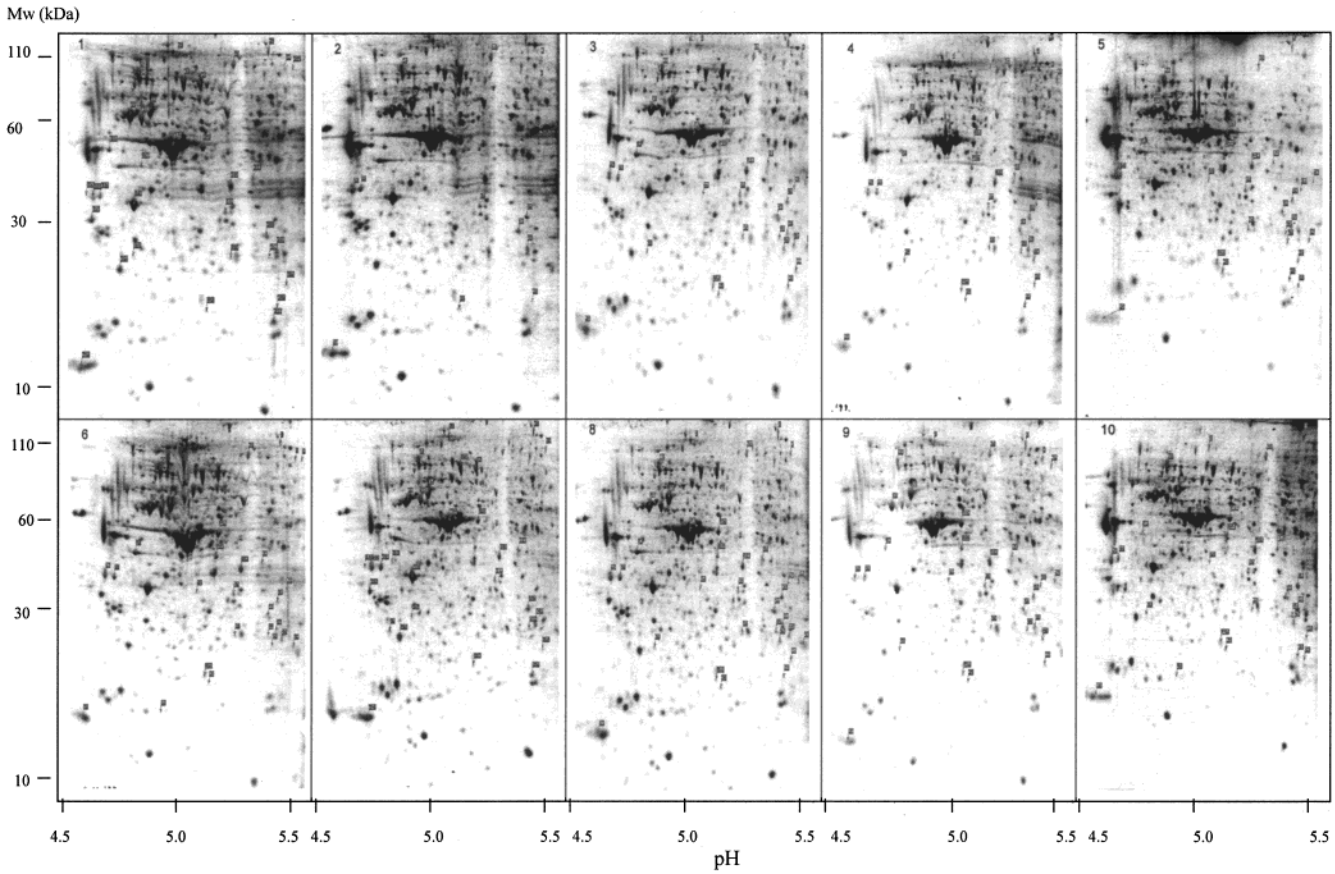

FIGURE 2: 2D gel electrophoresis of proteins from human lung fibroblast cells with/without stimulation with endothelin-1. New protein synthesis was detected by radioactive labeling with [^{35}S]methionine. Upper panel: unstimulated cells. Lower panel: stimulated cells. The elapsed time (minutes) following initiation of stimulation is indicated on the gels.

DISCUSSION

The set of proteins which show significant changes in protein synthesis over short time periods after endothelin stimulation (Table 1) involves proteins which have been implicated in a very wide range of different cellular and extracellular processes. This diversity presumably reflects the fact that the response to a mitogenic signal such as endothelin stimulation requires major changes in cellular function at many levels including intracellular changes connected ultimately with cell division, extracellular interactions with neighboring cells, and homeostasis at the organismal level. On the other hand, the number of proteins observed in the early response to endothelin stimulation is a very small subset of the total genomic repertoire, suggesting that the proteins that have been observed may have key roles in facilitating and directing this response.

As will be seen in the following, these observations may suggest new roles or reinterpretations of the roles of individual proteins in human lung fibroblast cells.

An important aspect of this study was the observation that changes in the fluxes of new protein synthesis were highly time dependent and that the induction of these changes occurred on a similar time scale to signaling responses observed by phosphorylation/dephosphorylation (*8*). This provides confidence that the observed changes in protein synthesis are a direct consequence of the signaling. It is also important to note that the identity of the proteins which show strong changes in the flux of new protein synthesis is strongly dependent on the time after stimulation (Figure 3). This is likely to also be a feature of methods such as expression profiling and emphasizes the need for explicit consideration of time in all experiments measuring cellular response. Indeed, in the future it may be possible to use quantitative statistical analysis of time series of the type demonstrated in this paper to unequivocally identify the groups of functionally related proteins and the kinetics of the controlling processes (*22*).

One of the most interesting groups of kinetically related proteins is the group shown in Figure 3E: Rab3A, Rab14 (long), annexin IV, Bcl2-related protein A1, translationally controlled tumor protein, and heat shock 70 kDa protein 5. Rab proteins are known to be important in intracellular vesicle trafficking (*23*). Rab14 has been observed in whole brain, spinal cord, heart, kidney, and lung cells, but there do not seem to be any studies of its physiological function nor any reports of variant isoforms. In contrast, Rab3A is one of the best studied of the many Rab proteins and is known to play a key regulatory role in Ca^{2+}-dependent exocytosis, particularly in neurotransmitter release from nerve terminals (*23*). The translationally controlled tumor proteins (TCTPs) are a highly conserved and abundantly expressed family of eukaryotic proteins that are implicated in both cell growth and the human acute allergic response but whose intracellular biochemical function has remained elusive. Very recently, on the basis of the solution structure of the TCTP from *Schizosaccharomyces pombe* (*24*), it has been proposed that the TCTPs form a structural superfamily with the Mss4/Dss4 family of proteins. These latter proteins bind to the

Table 1: Proteins Identified by Mass Spectrometry

spot	identified protein	NCBI entry	mass (Da) expected	mass (Da) measured[c]	p*I* expected	p*I* measured	no. of peptides[a]	Δmass[b]	% sequence
s1	vinculin	4507877	116 723	115 000	5.83	6.14	20	0.12	40
s2	splicing factor 3b, subunit 2	5803155	97 657	108 000	5.53	5.34	9	0.19	11
s3	glucosidase II	2274968	106 900	107 000	5.71	5.84	13	0.26	20
s4	nucleolar transcription factor 1 (UBF-1)	136652	89 406	91 000	5.63	6.27	13	0.11	19
s5	heat shock 70 kDa protein 5	386758	70 261	78 000	5.98	5.01	12	0.24	22
s6	annexin VI	113962	75 873	73 000	5.42	5.55	8	0.22	18
s7	filamin	938227	24 267	25 000	4.80	4.74	5	0.15	35
s8	vimentin	2119204	53 652	59 000	5.06	5.12	29	0.16	43
s9	tubulin β-1 chain	338695	49 759	57 000	4.75	4.85	5	0.14	21
s10	protein disulfide isomerase P5 precursor	5031973	46 171	56 000	4.95	5.03	10	0.09	33
s11	plasminogen activator inhibitor 2	1352712	46 597	47 000	5.56	5.56	5	0.05	12
s12	β-actin	4501885	41 737	42 000	5.29	5.30	14	0.12	51
s13	annexin I	113944	38 714	38 000	6.57	5.81	11	0.17	43
s14	transcription factor Sox5	5902114	38 848	40 000	6.53	6.00	10	0.16	25
s15	tropomyosin 2	4507649	32 990	35 000	4.63	4.55	6	0.15	19
s16	tropomyosin 4	136090	32 851	35 000	4.66	4.60	9	0.08	32
s17	tropomyosin 3	136085	32 819	33 000	4.68	4.65	5	0.06	18
s18	annexin V	189615	35 937	34 000	4.94	4.95	9	0.09	30
s19	AF091080	3859998	36 629	33 000	5.85	5.77	5	0.20	20
s20	annexin IV	1703319	35 883	33 000	5.84	6.41	5	0.14	21
s21	14-3-3 protein ϵ	5803225	29 174	32 000	4.63	4.60	6	0.04	32
s22	cytochrome P450-sce	189438	27 938	29 000	7.23	6.11	6	0.10	26
s23	Rab14 (long)	464559	23 927	25 000	5.85	5.80	6	0.29	12
s24	ornithine decarboxylase	852428	24 360	27 000	7.84	6.21	5	0.09	20
s25	translationally controlled tumor protein	136479	19 595	24 000	4.84	4.87	5	0.04	45
s26	Bcl2-related protein A1	4757840	20 132	21 000	5.32	5.60	5	0.21	24
s27	follistatin (fragment)	5453652	34 121	22 000	5.54	6.21	10	0.16	38
s28	Rab14 (short)	6563200	23 927	21 000	5.85	6.18	6	0.05	40
s29	nonmuscle myosin light chain alkali	127148	16 961	14 000	4.46	4.50	6	0.09	47
s30	nucleophosmin	825671	30 938	37 000	4.71	4.75	4	0.13	26
s31	Rab3A	4506367	24 984	25 000	4.85	4.90	7	0.14	33
s32	heat shock 70 kDa protein 8	5729877	70 898	71 000	5.37	5.40	12	0.13	26
s33	40S ribosomal protein SA	307105	32 854	48 000	4.79	4.75	6	0.12	34
s34	translation initiation factor IF3, subunit 1	4503511	28 990	26 000	4.74	6.15	8	0.08	28
s35	cyclin-dependent kinase inhibitor 1B	1168873	22 073	22 000	6.54	6.33	7	0.21	33

[a] Number of distinct peptides used to identify the protein. [b] The average deviation between the theoretical and observed masses of the peptides. [c] Mass information obtained after PAGE.

GDP/GTP free form of Rab proteins and have been termed guanine nucleotide-free chaperones. In this context, the present paper appears to be the first experimental observation of a possible functional linkage between Rab proteins and translationally controlled tumor protein. Furthermore, given the role of Rab3A in Ca^{2+}-dependent exocytosis and the fact that the TCTPs are also calcium binding proteins (*25*), it is intriguing that annexin IV is also included in this group of kinetically related proteins, albeit with changes in new protein synthesis opposed to those observed for Rab3A, Rab14, and TCTP (Figure 3E). The annexins are a family of calcium and phospholipid binding proteins whose physiological functions are poorly understood and for which many diverse functions have been proposed, including in vesicle trafficking and as intracellular ion channel regulators (*26*). Furthermore, new synthesis of heat shock 70 kDa protein 5 parallels that of annexin IV, suggesting that it might be especially important for folding of annexin IV. Finally, Bcl2-related protein A1 is a well-known antiapoptotic protein (*27*). In general, it seems likely that this group of proteins is involved in some form of vesicular trafficking which, despite the large changes in Rab3A, seems unlikely to be an exocytotic process and might be related to the endocytosis and recycling of endothelin receptor (*28*). However, this group of proteins might also be involved in cytoskeletal rearrangements given recent reports that Rab proteins may be involved in microtubules (*23*, *29*) and that TCTP has been reported to bind to microtubules (*30*).

A curious feature of the present experiments was that an isoform of Rab14 with a substantially lower M_r (s28, Table 1) and very different kinetic behavior (Figure 3D) was also observed. The very different kinetic behavior of the two isoforms of Rab14 presumably reflects regulation of the response to endothelin at the level of mRNA splicing. In this context it is interesting that the variant form of Rab14 was grouped kinetically with nucleophosmin (Figure 3D), which is an abundant, multifunctional nucleolar protein that has been reported to have a possible role in assembly and/or transport of ribosomes, molecular chaperone activity, and ribonuclease activity (*31*). Nucleophosmin has also recently been reported to be important in centrosome duplication via phosphorylation by cell cycle kinases (*32*, *33*), and it is therefore intriguing that cyclin-dependent kinase inhibitor 1B is also included in this set of kinetically related proteins. Since centrosome duplication is not important at the short times in our experiments, it appears that there are probably also other functions for this group of proteins. The present results also monitor a number of proteins which are generally connected with various aspects of RNA synthesis. These include proteins involved in transcription of rRNA genes (nucleolar transcription factor 1), RNA translation (eukaryotic translation initiation factor 3, subunit 1), or in RNA splicing

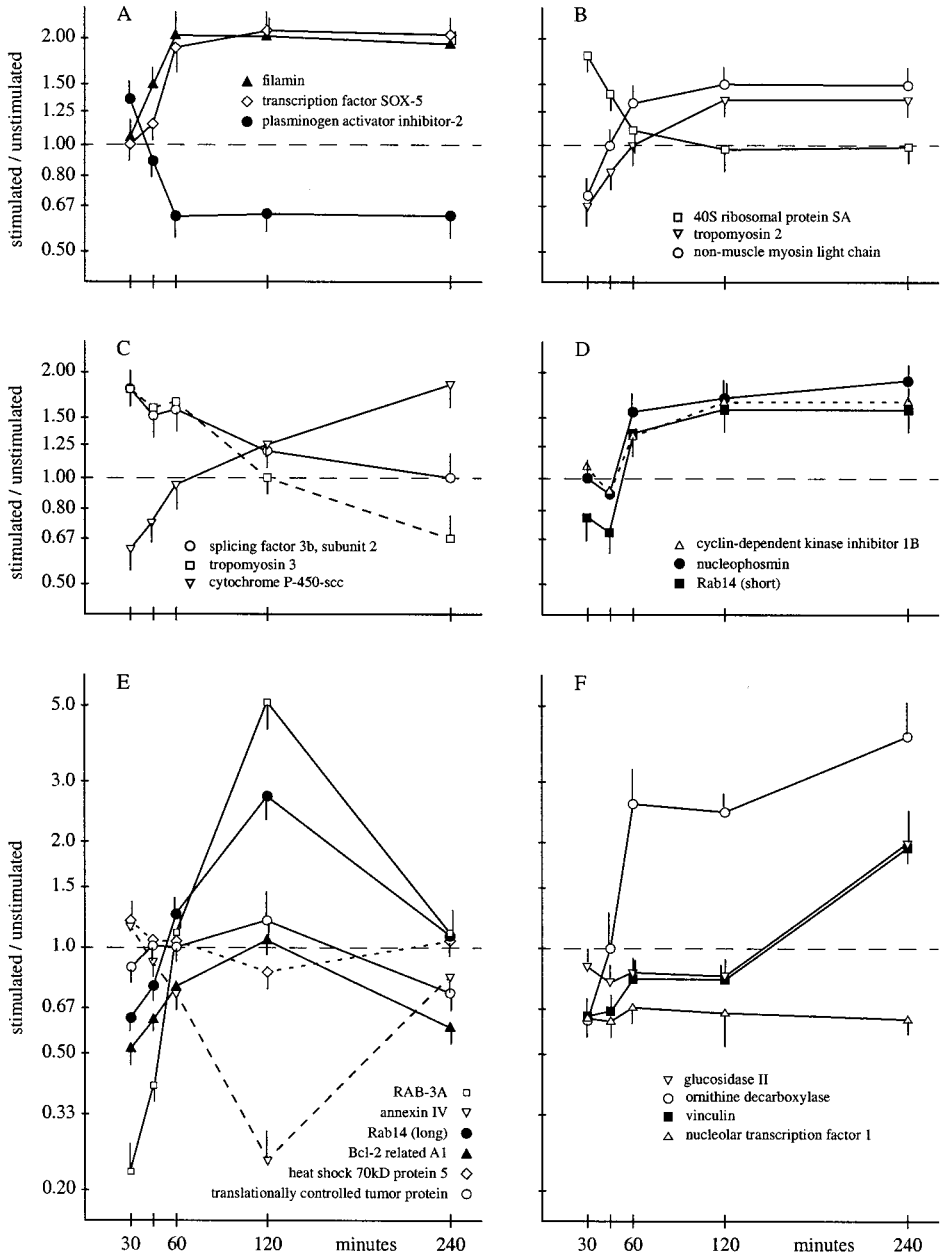

FIGURE 3: Time dependence of the ratio of new protein synthesis in stimulated/unstimulated cells plotted on a log scale as a function of time after initiation of stimulation with endothelin-1. Separate panels have been used to group proteins which show similar time dependence (panels A−E).

(splicing factor 3b, subunit 2). Nucleolar transcription factor 1 (upstream binding factor 1) is one of the essential components of the initiation complex for RNA polymerase I (*34*). For nucleolar transcription factor 1, the quantity of this protein (*34*), posttranslational phosphorylation by G1-specific cdk-cyclins (*35*), acetylation (*36*), and interactions with the product of the retinoblastoma susceptibility gene Rb (*37*) all appear to contribute to regulation of rRNA transcription. In the present experiments we observed that expression of nucleolar transcription factor 1 is downregulated by about 40% within 30 min of endothelin-1 stimulation and remains suppressed (Figure 3F). This would seem to be consistent with reports that this protein is inactive in early G1 phase (*38*). Very different behavior is shown by splicing factor 3b, subunit 2, which shows upregulation of about 80% 30 min after stimulation with endothelin-1 followed by a slow decrease to the synthesis levels observed in unstimulated cells (Figure 3C). Similar behavior is observed for eukaryotic translation initiation factor IF3, subunit 1. It is particularly interesting that we observe changes in synthesis for single proteins that are components of large multiprotein complexes, e.g., only subunit 1 of the 10 or more different proteins comprising eukaryotic translation initiation factor 3 and only subunit 2 of the 4 different proteins comprising splicing factor 3b. This seemingly anomalous result might be explained if these proteins have special roles in initiation or localization of such complexes. It is also interesting that 40S ribosomal protein SA shows an initial upregulation of about 80% at 30 min followed by a slow decay to normal synthesis levels (Figure 3B). This seems likely to be connected with its alternative role as a laminin binding protein at the cell surface (*39*) rather than as a component of the ribosome. It may be noted that upregulation of this protein is observed in a variety of different types of cancer (*40*).

The proteins included in Table 1 also include a large number of proteins involved in cytoskeletal interactions. This should not be surprising since cytoskeletal interactions are known to be important in a wide variety of cellular processes including signaling systems, cell−cell interactions, and mitosis. Thus the proteins in Table 1 include proteins involved in all three general types of cellular filaments, e.g., tubulin (tubulin β-1), intermediate filaments (vimentin), and microfilaments (vinculin, filamin, β-actin). An interesting feature of the present experiments is that although there are changes in new protein synthesis for such a variety of proteins, within any single family of molecules, only a few of the known protein species show changes in protein synthesis. Thus, for example, of the 13 known types of annexin, only 4 types, annexins II, IV, V, and VI, showed changes in protein synthesis. Similarly, of 10 reported β-tubulin isoforms, only the tubulin β-1 chain was observed in the endothelin-induced protein synthesis changes. Furthermore, related proteins from the same family may show very different kinetic behavior (see tropomyosins 2 and 3 in Figure 3B,C). These results provide confirmation of the specificity of the response to endothelin stimulation, but further work will be needed to ascertain whether the selection of individual proteins from extensive protein families is dictated by the specific stimulatory signal (endothelin-1), the specific cell type (human lung fibroblasts), or both.

One of the more unexpected results of the present experiments was the observation of the upregulation of the transcription factor Sox5 (Figure 3A). This protein has previously only been reported in the context of spermatogenesis with localization in the adult testis (*41*, *42*). Although the Sox transcription factors have been widely studied, this has usually been in the context of cellular differentiation and embryonic development. Among the hundreds of papers on Sox transcription factors, only a few deal specifically with Sox5, and there do not seem to be any reports dealing with involvement of Sox5 in response to a signaling process or with a role of Sox5 in fibroblasts. Neither is there any evidence suggesting why Sox5 might be functionally linked with filamin or plasminogen activator inhibitor 2 (Figure 3A). At present we speculate either that Sox5 may have other types of roles or that replication of differentiated cells involves activation of cellular systems to maintain the identity of the differentiated cell type and that Sox5 is particularly important in maintaining differentiation of human lung fibroblasts. If this is a general phenomenon, it may be that experiments of the type presented in this paper would provide an alternative method for investigating the involvement of Sox and/or other transcription factors in maintenance of differentiation of different cell types.

Although it is not possible to comment here on all of the changes in new protein synthesis which have been observed, the above examples may serve to show that these experiments have revealed changes in unexpected proteins and are suggestive of unanticipated roles for some proteins. Together with the kinetic groupings, which probably reflect functional links in most cases, the present experiments provide a rich set of suggestions for further investigation. A few proteins have been observed for which either the variant observed here seems not to be present in sequence databases (follistatin fragment, short form of Rab14), previously discussed functional roles do not give any clear relationship to endothelin stimulation (e.g., Sox5, follistatin fragment), or do not have any known functional role at present (AF091080, Table 1). All of these are clearly candidates for further investigation, as are the further 12 proteins detected for which sequences seem not to be available in present databases.

Interestingly, the proteins which showed strong changes in new protein synthesis in the present experiments were different from those observed in previous studies of endothelin stimulation of mouse fibroblast cells (*43*), astrocytes (*44*, *45*), and rat mesangial cells (*46*). These earlier studies investigated endothelin effects on cell cycle progression and showed strong increases of cyclins D1 and D3, cdk4 kinase, p16INK4, p21cip1, and the phosphorylated form of pRb. Changes in the expression of several protooncogenes (c-fos, c-jun, and c-myc from vascular smooth muscle cells) following endothelin stimulation have also been reported previously (*14*−*17*). The different proteins may be a consequence of the different cells but probably also reflect the different time scales of the experimental protocols. Whereas we have observed appreciable differences in rates of new protein synthesis by using pulse stimulation and labeling with short observation times, the previous experiments measured changes in total protein concentrations of maximally 2−5-fold following 4−24 h of constant exposure to endothelin (*14*−*17*, *43*−*46*). This latter type of experiment therefore measures adaptation of the cells to long-term exposure to endothelin and hence may reflect generalized changes consistent with cell growth rather than new protein synthesis that is an early consequence of endothelin stimulation. As we have discussed in more detail elsewhere (*6*), detection of cellular responses at short times with pulsed stimulation is likely to be more informative about the immediate interconnectivities of cellular pathways. Finally, it may be commented that, apart from the 25-fold change in the rates of new protein synthesis for Rab14 (long) observed over the time course of our experiments, all of the other proteins showed changes of less than 5-fold and many were less than 2-fold (Figure 3). This seems to be consistent with the growing number of expression profiling experiments that typically observe limited changes in a large number of mRNAs rather than on/off behavior of a few genes (*47*).

CONCLUSION

The use of proteomics to monitor gene expression has several potential advantages. First, monitoring of mRNA levels by expression profiling (*1*) may not give quantitative measurements of new protein synthesis; e.g., cases are known where 40-fold changes in mRNA concentrations do not lead to any change in protein concentration (*4*). These types of problems, which may complicate interpretation in physiological terms, can be directly circumvented by the pulse radioactive labeling of newly synthesized proteins employed in the present work. In the present initial study, we have used autoradiography with film detection to detect newly synthesized proteins and conventional MALDI-TOF mass spectrometers to identify these proteins. The major limitation in the present experiments was the mass spectrometers available to us, which necessitated the use of low femtomole protein quantities for the protein identifications. In this context it is important to note that more sophisticated MS spectrometers are already capable of performing these types of analysis with more than 1000-fold smaller protein samples

(*48*). Furthermore, multiphoton detection methods to detect radioactively labeled proteins at subattomole quantities on 2D gels, with linear responses over at least 7 orders of magnitude of concentration of different proteins, are in advanced stages of development (www.biotraces.com). This suggests it should be possible to extend the present experiments to observe newly synthesized proteins at levels of less than 10 copies/cell, to extend the experiments to shorter initial time periods, to improve the quantitation of these experiments over large dynamic ranges, and at the same time to use fewer cells. However, the fact that only about 70 proteins were observed in the present experiments strongly suggests that the number of proteins sensitive to pulsed endothelin stimulation will still remain a small subset of the total number of proteins extant in the fibroblasts or of the total number of proteins in the genome.

A second potential advantage of the proteomic methods is that they provide simple methods for monitoring all proteins as synthesized and/or modified without any preknowledge of the proteins that need to be monitored. This seems to be particularly important in view of recent reports that less than half of human genes have so far been reliably identified by genome sequencing efforts (*49*). We would also contend that it would have been virtually impossible to predict the set of 35 identified proteins which showed substantial changes in protein synthesis in the present study and that it would therefore have been very difficult to design suitable nucleotide arrays to monitor all of these proteins. The difficulty of constructing appropriate nucleotide arrays is further emphasized by the fact that although at present we have only characterized the proteins at the DNA level, i.e., identification of the gene from which they are expressed, several clear cases of proteins which do not correspond to the full gene sequences or are splice variants were detected, e.g., follistatin fragment, the short form of Rab14, nucleolar transcription factor 1, and vinculin. The ubiquity of splice variants and posttranslational modifications is a thorny problem for expression profiling (*1*). As has been discussed in some detail elsewhere (*6*), the monitoring of proteins at the DNA level (the gene expressed), the RNA level (splice variants), and the phenotypic level (including posttranslational modifications such as proteases, phosphorylation, etc.) is crucial for understanding cell function, is rapidly becoming very routine in MS analyses of proteins, and can be readily included in proteomic experiments which monitor expression. Another advantage of the present proteomic methods is that, by using preselection of the interesting proteins by pulse radiolabeling, the magnitude of the necessary analytical work is dramatically reduced. Although we estimate that more than 3000 different proteins were present on the 2D gels used in the present experiments, only about 70 needed to be analyzed in detail. There are already numerous examples of cases where cellular response to a given stimulation involves only a small subset of total cellular proteins, and the present experiments provide effective preselection procedures for taking advantage of this characteristic in analyzing new protein synthesis. Furthermore, we would suggest that functional proteomic experiments of the type presented in this initial study are competitive with expression profiling methods for many applications and may be more immediately accessible to usual cell biology laboratories.

Finally, a major advantage of these types of functional proteomic methods is that they readily lend themselves to simultaneous measurement of other essential processes. The complex kinetic results observed in the present paper are in accord with the fact that new protein synthesis in response to endothelin stimulation is intimately linked with other processes occurring at the purely protein level, e.g., the phosphorylation cascades of the signaling processes which occur concurrently for stimulation of fibroblast cells with endothelin (Soskic and Godovac-Zimmermann, unpublished results). The ultimate advantage of the proteomic methods may lie in the ability to simultaneously observe regulatory processes at both the purely protein level and the level of gene expression.

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