Rapid Changes in the Phosphoproteome Show Diverse Cellular Responses Following Stimulation of Human Lung Fibroblasts with Endothelin-1

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ABSTRACT: The rapid phosphorylation and dephosphorylation of a variety of proteins downstream of the endothelin receptors A and B was investigated following stimulation of human lung fibroblasts with endothelin-1. Changes in the phosphorylation of proteins involved in the cell cycle, cytoskeleton, membrane channels, transcription, angiogenesis, and metabolism were observed. From observed changes in protein phosphatase 2A, CDC25 A, and caspase-2 precursor, a model for the promotion of cell cycle progression by ET-1 stimulation is proposed. This may offer insights into the mechanisms by which ET-1 exerts its mitogenic effects. The identities of the other proteins phosphorylated within 2 min of stimulation indicate that endothelin-1 also rapidly engages a diverse variety of other cellular responses.

Endothelin is a strong vasoconstrictor with physiological effects on cellular development, differentiation, vasoconstriction, and mitogenesis (*1*, *2*). These multiple physiological effects are mediated via the G-protein-coupled receptors ETA and ETB (*3*, *4*). Endothelin-1 has been shown to stimulate mitosis of many different cell types, including smooth muscle (*5*), breast stoma (*6*), kidney mesangial (*7*), and rat-1 fibroblast cells (*8*). It is likely that endothelin acts as a mitogen in many cell types since ET-1 stimulation has been shown to activate a variety of promoters of cell division. One such promoter is the enzyme MAP kinase (*9*−*11*), which is activated by tyrosine phosphorylation (*12*). The activation of this enzyme is probably mainly due to activation of upstream PKC via the inositol phosphate pathways. Activation of these pathways can lead to the induction of the proto-oncogenes c-fos and c-myc (*13*). In addition, interactions that mimic EGF binding have been shown between ET receptors and tyrosine kinase receptors (*14*−*16*). Apart from indirect action on growth factor receptors, ET-1 has been shown to increase the production of growth factors such as VEGF in osteoblast cells (*17*).

In addition to promoting cell proliferation, there is also evidence that ET-1 may play a role in cell survival. Like cell proliferation, inappropriate cell survival can lead to the development of cancers or other disorders. Experiments with cell lines from human colon carcinoma and glioblastomas have shown that ET-1 may be an anti-apoptotic factor (*18*). The literature suggests that there are several mechanisms by which endothelin can increase cell growth and division. While there may be a physiological role for these mechanisms, they could also cause or maintain a cancer if not properly controlled (*19*). Alternatively, endothelins could contribute to the development of cancer by stimulating the growth of blood vessels supplying tumors. ET-1 has been shown to encourage the growth of new blood vessels by direct action through the ETB receptor (*20*). Secretion of ET-1 by cells may also be autostimulatory, setting up an autocrine loop (*21*). In addition, ET-1 can indirectly stimulate neovascularization via the ET-1 receptor by stimulating the release of VEGF (*22*, *23*).

The diversity of the biological effects of ET-1 suggests that many intracellular pathways may be stimulated directly or indirectly via the ETA and ETB receptors. In the present paper, we show that rapid changes in protein phosphorylation upon stimulation of human lung fibroblasts with endothelin-1 are observed for a variety of proteins implicated in very different physiological processes. We propose a new model for the action of ET-1 in the promotion of cell cycle progression, which may offer insights into the mechanisms by which ET-1 exerts its mitogenic effects. In the companion paper (*24*), we show that the ETA and ETB receptors show a multiplicity of differently posttranslationally modified forms in both the stimulated and the unstimulated states and that these isoforms change rapidly following stimulation with ET-1. We suggest that the diversity of the forms of these receptors may be coupled to the diversity of responses elicited by ET-1 in human lung fibroblasts (*24*).

MATERIALS AND METHODS

Materials. The cell line CCD33Lu was from the European Collection of Cell Cultures (Salisbury, UK). Trypsin-EDTA, antibiotic/antimycotic, minimal essential medium (MEM),[1] Hank's balanced saline solution (HBSS), nonessential amino acids (NEAA), and L-glutamine were from GibcoBRL (Eggenstain, Germany). FBS was from PerbioScience (Tattenhall, UK). HPLC-grade acetonitrile, methanol, and acetic acid were from BDH (Poole, UK). Duracryl (30%

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[1] Abbreviations: MEM, minimum essential media, with Earle's salts, w/o L-Glu; NEAA, nonessential amino acids; FBS, fetal bovine serum; ET-1, endothelin-1; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESI-ion trap MS, electrospray ionization-ion trap mass spectrometry.

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acrylamide, 0.8% BIS) was from Genomic Solutions (Huntingdon, UK). DMF was from Fluka Chemie (Gillingham, UK). Complete miniprotease inhibitor tablets were from Boehringer (Mannheim, Germany). Modified trypsin was from Promega (Madison, WI). All other chemicals were from Sigma-Aldrich (Gillingham, UK) and of the best grade available.

Cell Culture. A human lung fibroblast cell line (CCD33Lu) was grown in MEM containing 2 mM L-Glu, 1% NEAA, 0.8% antibiotic/antimycotic solution, and 10% FBS. Cells were grown at 37 °C, 5% CO_2 in 75 cm^2 dishes.

Preparation of Stimulated and Unstimulated Cell Samples. Cells were serum-starved overnight in 5 mL of MEM containing 200 mM L-glutamine, 200 mM NEAA, and 0.5% FBS. The following day the dishes were divided into two groups—stimulated and unstimulated. The 1 μM ET-1 in PBS was added to the stimulated dishes to a final concentration of 50 nM, and an equivalent volume of PBS was added to the unstimulated group. All dishes were then incubated for 2 min at 37 °C and transferred immediately to ice. The medium was aspirated from both groups, and the cells were washed twice with 5 mL of ice-cold PBS containing protease inhibitors (complete minitablets).

A total of 0.5 mL per dish of ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 1% Nonident NP40 v/v, 1% Triton X-100 v/v, 1 mM Na_3VO_4, 5 mM NaF, 5 mM β-glycerophosphate, 5 mM EDTA, 1 complete miniprotease inhibitor cocktail tablet per 100 mL) was added, and the cells were scraped on ice to release them from the dish and then sonicated for 30 0.5-s bursts. The samples were spun down at 13 000 rpm for 20 min at 4 °C. The pellet was discarded, and the supernatant, containing solubilized cellular proteins, was made up to 0.2 M NaCl and then incubated under rotation with 200 μL of 50% Sepharose 4B at 4 °C. The mixture was then spun down (2 min, 3000 rpm, 4 °C), and the pellet was discarded. A solution of 1% low melting point agarose was added to the supernatant to a final concentration of 0.05% agarose. The sample was vortexed briefly, then spun down at 3000 rpm, 2 min, 4 °C, and the pellet was discarded.

Isolation of Phosphorylated Proteins. The sample was incubated under rotation for 3 h at 4 °C with 250 μL of 50% antiphosphoserine agarose (Sigma-Aldrich, Gillingham, UK). Empty microcolumns (Mobitec, Goettingen, Germany) were loaded with a maximum of 700 μL of sample/agarose and spun briefly to about 5000 rpm. Columns were rapidly washed three times with 0.5 mL of ice-cold lysis buffer and once with 0.5 mL of ice-cold 10 mM Tris-HCl pH 7.4. The agarose was immediately resuspended in ice-cold IEF buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% Triton X-100, 10 mM Tris base, 0.8% Pharmalyte v/v (4−7 or 3−10 according to the strip used, Amersham, UK), and one complete miniprotease inhibitor cocktail tablet per 100 mL) to give a total volume of 300 μL per gel and incubated at room temperature for 1 h to elute the proteins from the agarose. Isolation using antiphosphothreonine and antiphosphotyrosine antibodies was performed in exactly the same way on separate samples.

Isoelectric Focusing. DTT was added to the sample to a concentration of 65 mM. The 13 cm linear gradient isoelectric focusing strips, pH 3−10 or 4−7 (Amersham Pharmacia) were reswollen overnight in 300 μL of sample per strip and covered by a layer of mineral oil. Strips were transferred to strip holders and covered with 2 mL of mineral oil. Focusing was carried out on a Amersham IPGPhor system, using a step-and-hold protocol of increasing voltage as follows: 30 V for 30 Vh, 300 V for 150 Vh, 1000 V for 500 Vh, 3000 V for 3000 Vh, 8000 V for 60 000Vh, and 2000 V for 12−24 h. The IEF strips were equilibrated prior to second dimension electrophoresis in 2 mL of equilibration buffer (30% glycerol v/v, 2% SDS w/v, 6 M urea, 0.5 mM Tris-HCl, pH 6.8) containing 0.1 M DTT, then in 2 mL of equilibration buffer containing 0.2 M for 20 min, for 20 min each at RT.

Second Dimension Electrophoresis. The 13 cm × 16 cm × 1 mm gels were cast with an 11% polyacrylamide mixture, using the Hoefer-Dalt system (AP Biotech). Equilibrated IEF strips were loaded horizontally and sealed in place with 0.5% agarose in Laemmli running buffer, containing bromphenol blue. A small well was left in the agarose alongside the strip, and molecular weight markers (10 kD protein ladder, Biorad (Hemel Hempstead, UK)) were added and sealed with agarose. Gels were run vertically at 300 V for 3−4 h in Laemmli running buffer cooled to 8 °C and subsequently silver-stained.

In-Gel Digestion. Spots appearing in only one set of gels (stimulated or unstimulated) were identified, and a small square of gel from each spot was digested as follows. The gel was reswollen in 50 mM NH_4HCO_3 for 3 × 15 min, then destained in 50 mM sodium thiosulfate and 15 mM potassium ferricyanide. After washing in 3 × 100 μL of ddH_2O, the gel was dried for 3 × 10 min in AcN. The liquid was removed, and the gel was dried in a speedvac for 30 min. The dry gel was incubated at 56 °C in 100 mM NH_4HCO_3 containing 10 mM DTT for 30 min, then dehydrated in AcN as previously stated, and dried in the speedvac for 30 min. The gel pieces were incubated overnight at 30 °C in 5 μL of modified trypsin (75 ng/μL in 50 mM NH_4HCO_3).

MALDI-TOF Mass Spectrometry. A total of 0.5 μL of the tryptic digest was applied to the target plate and allowed to air-dry, then overlaid with 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% AcN, 0.1% TFA v/v. Spectra were taken on a Biflex III MALDI-TOF spectrometer (Bruker, Germany), which was calibrated with a mixture of five known peptides. The spectrometer was usually in reflector mode, detector voltage 1.8 kV, laser attenuation 70%, although these parameters were varied to obtain better spectra. Peaks were identified manually using Bruker DataAnalysis software. The peaks identified were compared with theoretical protein digests generated by the Protein Prospector MS-Fit tool (http://www.jpsl.ludwig.edu.au), searching the Swiss-Prot database (www.expasy.ch) to identify the protein spots by mass fingerprinting.

ESI-Ion Trap Mass Spectrometry. Tryptic digest samples were diluted 1:10 in 50% methanol, 0.5% acetic acid. Samples were subjected to MS/MS analysis using a Finnigan-Matt LCQ. Collision energies ranged between 25 and 50%. Spectra were compared to theoretical fragmentations of the peptides identified by MALDI-TOF analysis, using the MS-Product tool at Protein Prospector (http://www.jpsl.ludwig.edu.au).

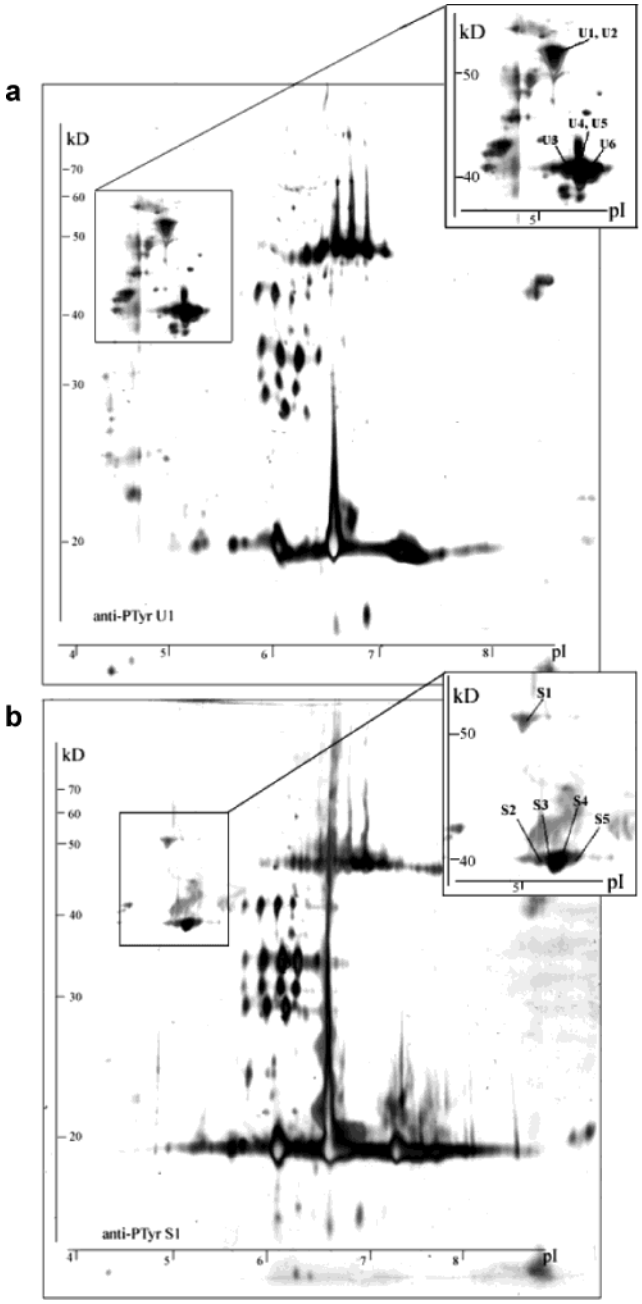

FIGURE 1: 2-D PAGE separation of phosphorylated proteins recovered from the anti-phosphotyrosine agarose column for (a) unstimulated lung fibroblast cells and (b) cells stimulated for 2 min with 50 nM ET-1. Inserts are expanded plots at pI range 4.5−5.5 and MW 37−62 kDa. The intense spots observed at low M_r (20 000) arise from leakage of the antibody from the agarose column.

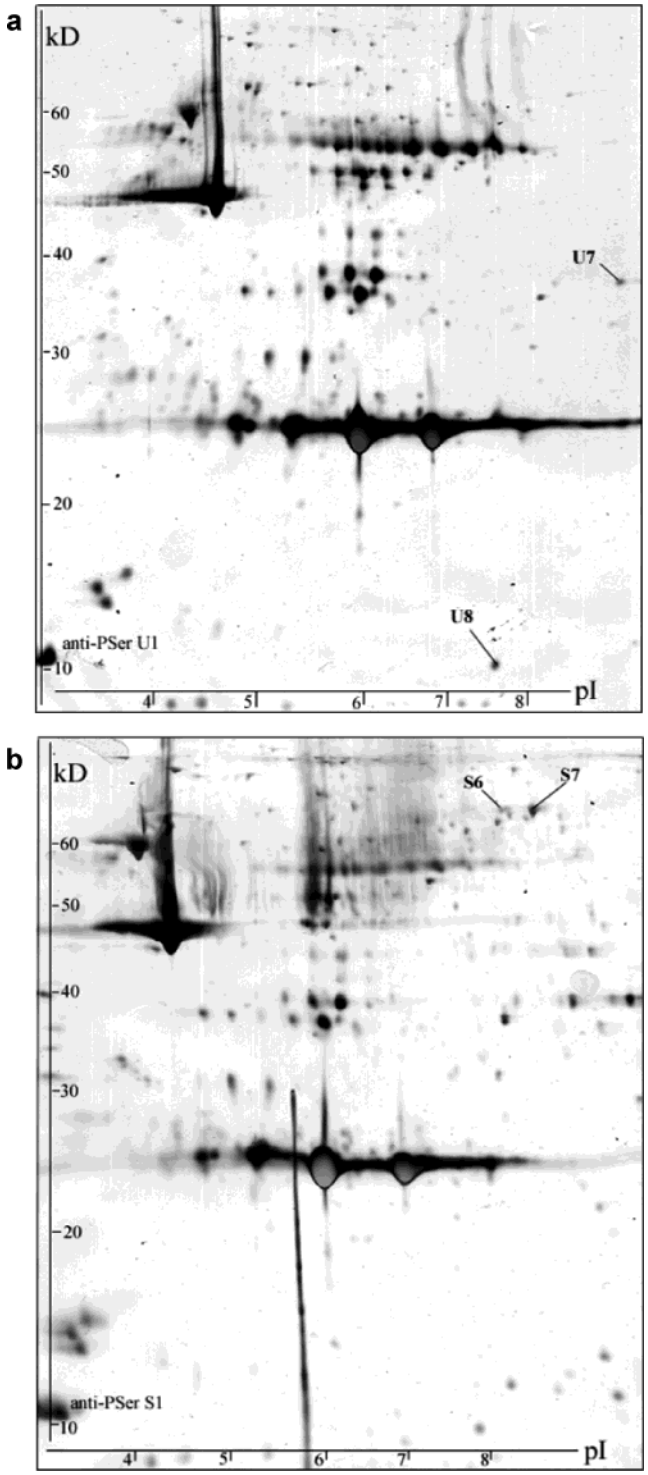

FIGURE 2: 2-D PAGE separation of phosphorylated proteins recovered from the anti-phosphoserine agarose column for (a) unstimulated lung fibroblasts and (b) cells stimulated for 2 min with 50 nM ET-1.

RESULTS

Protein phosphorylation is a ubiquitous cellular control mechanism that is thought to involve up to 30% of all cellular proteins. Studies of phosphorylation/dephosphorylation in response to cellular stimulation therefore provides an efficient means for investigating the involvement of different cellular pathways in the response of cells to external stimulations. In this study, human lung fibroblasts were stimulated with endothelin-1, and after a time interval of 2 min, proteins phosphorylated on tyrosine, serine, and threonine were individually isolated. The separation of the phosphoproteins into these three classes was carried out using a new isolation procedure developed in our labs that involves the use of agarose columns with attached anti-phospho-Tyr, -phospho-Ser, and -phospho-Thr antibodies. A very short time of exposure to ET-1 was used in these experiments to detect initial responses of the lung fibroblast cells.

Each of the three classes of extracted phosphoproteins was separated using a 2-D PAGE gel over the pI range 3−10 (Figures 1, 2, and 4). For proteins extracted with the antiphosphoserine antibodies, a further 2-D gel covering the pI range 3−7 was used to improve the separation of proteins

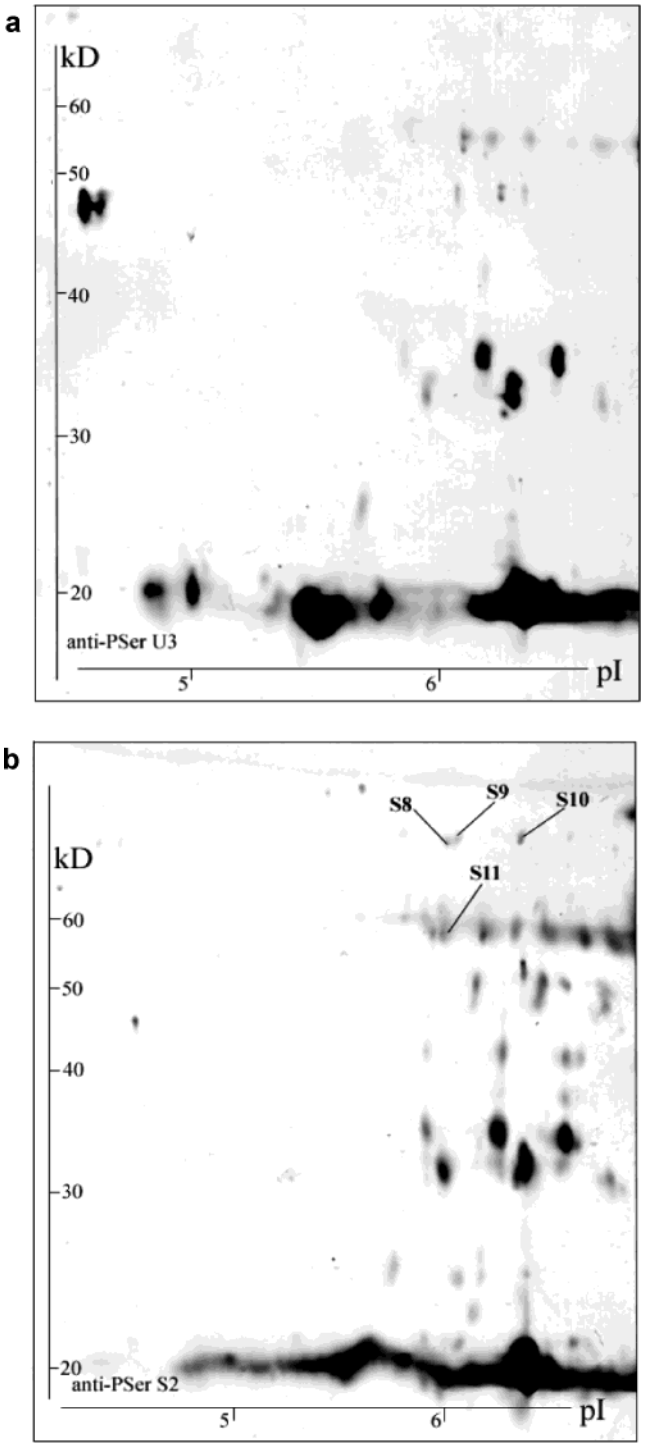

FIGURE 3: 2-D PAGE separation over pI 4−7 of phosphorylated proteins recovered from the anti-phosphoserine agarose column for (a) unstimulated lung fibroblasts and (b) cells stimulated for 2 min with 50 nM ET-1.

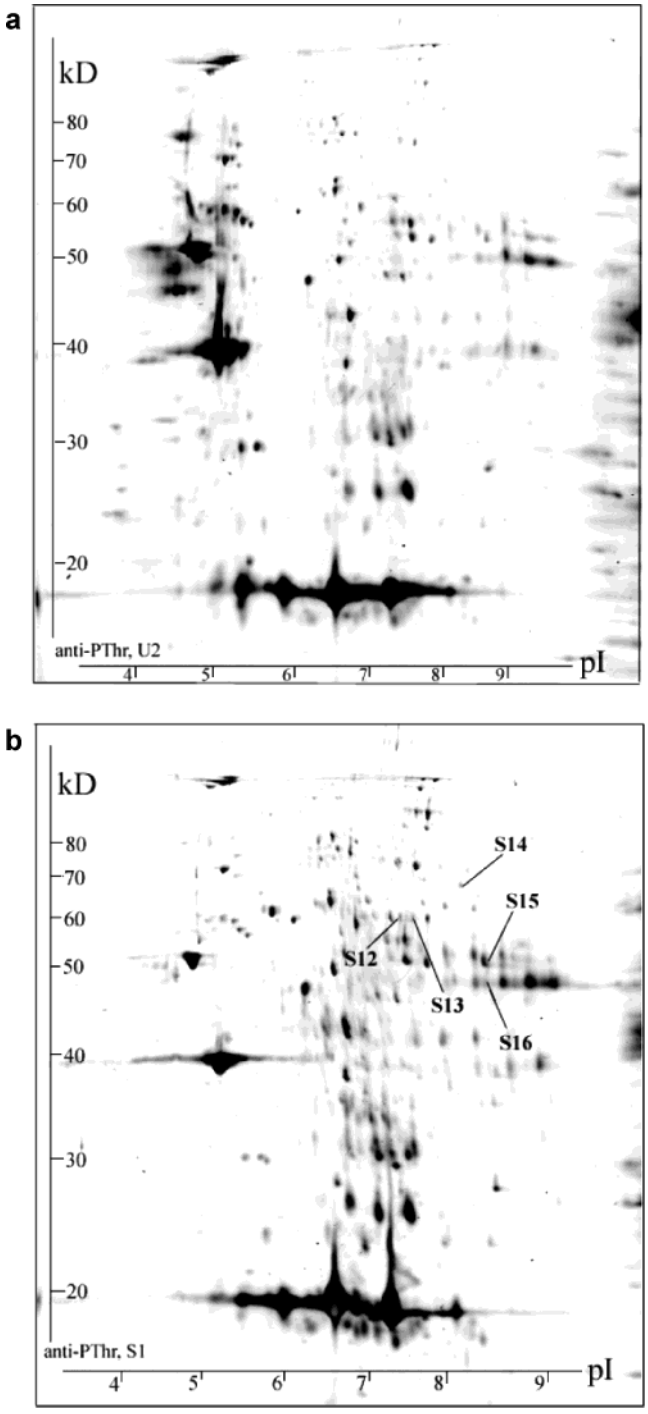

FIGURE 4: 2-D PAGE separation of phosphorylated proteins recovered from the anti-phosphothreonine agarose column for (a) unstimulated lung fibroblasts and (b) cells stimulated for 2 min with 50 nM ET-1.

containing phosphoserine (Figure 3). Comparison of these gels to 2-D gels run with total cellular protein extracts indicated that the extraction of the phosphoproteins led to substantial simplification of the protein patterns on the gels (not shown). There was also substantial enrichment of the amounts of phosphoproteins on the gels, and low abundance phosphoproteins that were not observable on 2-D gels of total protein extracts could be observed after fractionation.

The 2-D gels showed different numbers of proteins (Figures 1−4) with the smallest number of proteins being observed for the phosphotyrosine proteins. This is consistent with the observation that at the amino acid level, the distribution of phosphorylation in normal vertebrate cells is roughly 0.05% phosphotyrosine, 10% phosphothreonine, and 90% phosphoserine (*25*). Interestingly, at the protein level, there seem to be about as many proteins with phosphothreonine as phosphoserine (Figures 2−4). Many cellular proteins are phosphorylated at more than one site and often on more than one type of amino acid. Thus, some proteins phosphorylated on Tyr (Figure 1) also appear on the 2-D gels for proteins isolated with the anti-Ser or anti-Thr antibodies

Table 1: Identification of Phosphorylated Proteins from Human Lung Fibroblasts by MALDI-TOF and ESI-Ion Trap Mass Spectrometry

				mass (Da)		pI			
functional group	protein	spot	Swiss-Prot entry	exp.	obsd (approx)	exp.	obsd (approx)	% sequence	modification obsd
structural	actin	U3−6, S2−5	P02570 P02571	41 005	35−45 000	5.31	5.3	54	dephosphorylated, Tyr
	vimentin	U1−2, S1	P08670	53 554	50−55 000	5.03	4.9	79	dephosphorylated, Tyr
	T-plastin	S8	P13797	70 436	70−75 000	5.52	6.2	18	phosphorylated, Ser
membrane	adaptor-related protein complex 1, γ 2 subunit	S11	O75843	87 116	55−60 000	6.09	6.1	10	phosphorylated, Ser
	stomatin	S16	P27105	31 731	40−50 000	7.71	8.7	24	phosphorylated, Thr
	voltage-gated potassium channel protein KV3.4	S14	PQ03721	64 527	60−70 000	6.69	8.5	15	phosphorylated, Thr
enzymes	GFAT-1	S10	Q06210	76 747	70−75 000	6.39	6.5	29	phosphorylated, Ser
	cytochrome P450	S6, S13	Q16850	56 600	60−70 000	9.41	8.2	9	phosphorylated, Ser and Thr
					60−70 000		8.0	19	
growth and division	PP2A, delta subunit (B), regulatory subunit, delta-3 isoform	S7	Q14738	58 453	60−70 000	6.32	8.5	28	phosphorylated, Ser
	dual specificity phosphatase CDC25A (M-phase inducer phosphatase 1)	S12	P30304	58 797	55−65 000	6.35	7.9	11	phosphorylated, Thr
	caspase-2 precursor (procaspase-2)	U7	P42575	48 855	35−45 000	6.31	9.0	13	dephosphorylated, Ser
	Kruppel-related zinc-finger protein 2 (HRK-2)	U8	P10073	12 067	10−15 000	8.72	8.0	82	dephosphorylated, Ser
other/unknown	angiopoietin-related protein 2 [precursor]	S15	Q9UKU9	57 104	45−50 000	7.23	8.7	17	phosphorylated, Thr
	mosaic protein LGN	S9	P81274	75 802	70−75 000	6.03	6.25	11	phosphorylated, Ser

(Figures 2−4). Overall, about 1500 distinct spots could be detected on the gels with silver stain. This is good evidence for the enrichment achieved by the present fractionation method since earlier studies using total cellular protein extracts were only able to detect about 500 phosphorylated proteins even though more sensitive detection with antibodies was used (*26*).

Comparison of 2-D gels obtained for phosphoproteins extracted with/without stimulation of the fibroblasts with ET-1 showed substantial changes in the patterns of phosphorylation for all three classes of phosphoproteins (Figures 1−4). In these experiments, our goal was to detect proteins that showed major changes in phosphorylation within 2 min of exposure to ET-1 with a view to establishing what kinds of cellular pathways are directly influenced by the stimulation of ET receptors. Among the proteins observed on the various gels, there were a number of spots (indicated in Figures 1−4) that appeared to show 100% changes in the observed phosphorylation within 2 min of stimulation. These spots were either observable as phosphorylated proteins only after stimulation or disappeared completely from the phosphorylated proteins after stimulation. Although there were other spots that were much more intense and showed greater absolute changes in intensity on the gels (Figures 1−4), we have first analyzed those proteins showing apparent on−off behavior within 2 min of stimulation.

Nine of these proteins have been identified by mass spectrometry. For most of them, the measurement of peptide masses by MALDI-TOF MS following tryptic fragmentation gave sequence coverage greater than 20%, which is generally sufficient to obtain reliable identifications. Where sequence coverage was less than 20% by MALDI-TOF MS, nanospray MS/MS peptide sequencing was used to confirm the identifications. These proteins are shown in Table 1, grouped according to their likely roles within the cell. In addition, for certain spots arising from actin and vimentin, there was a sharp change in the amounts of these proteins on the gels of the phosphotyrosine proteins before and after stimulation (Figure 1), and these have also been included in Table 1. Although the MS data collected was primarily aimed at obtaining protein identifications rather than at the characterization of covalent structure over the full protein sequences, a number of phosphorylated peptides were sequenced by MS to confirm the presence of phosphorylated sites consistent with the selectivity of the different antibodies. For example, after stimulation with ET-1, the peptide 18-QCCVLFDFVpSDPLpSDLK-34 from PP2A was found to be phosphorylated at Ser-27 and Ser-31, while the peptide 278-pSQEEpSPPGpSpTKRR-289 from cdc25A was found to be phosphorylated at Ser-278, Ser-282, Ser-286, and Thr-287. Similarly, prior to stimulation, the peptide 61-VGSF-SQNVELLNLLPK-76 from procaspase-2 was phosphory-

lated on either Ser-63 or Ser-65. These peptides are consistent with the phosphorylation patterns observed on the different gels following stimulation (Table 1), but at present, we cannot exclude other phosphorylation sites in these proteins.

DISCUSSION

Although the idea of total proteomics is seductive, so far the available proteomics methods for resolution and detection of proteins have not been sufficient for observing all cellular proteins in single experiments. This has led to attempts to fractionate total cellular proteins in various ways so that subsequent display and identification by proteomics methods can be done with protein samples of manageable complexity. Phosphorylated proteins are a particularly interesting subclass of total proteins because phosphorylation is intimately connected with many cellular regulation mechanisms. However, analysis of the phosphoproteome has faced technical difficulties, including that phosphoproteins may be of low abundance, that the same protein may be phosphorylated in different ways, and that these proteins may only be transiently phosphorylated. The large number of phosphorylated proteins that could be observed in the present experiments indicates that the methods we have developed to separate phosphorylated proteins from nonphosphorylated proteins and to fractionate the phosphoproteins according to phosphorylation on Tyr, Thr, or Ser represents an important advance in the analysis of the phosphoproteome. Although we have so far only identified by MS a small number of phosphoproteins that showed on−off behavior following stimulation with ET-1, the identity of these proteins already provides interesting new information about signaling induced at endothelin receptors.

It is important to note that most of the proteins listed in Table 1 have not previously been shown by experiment to be involved in signaling at endothelin receptors. However, as shown in Table 1, many of these proteins can be roughly classified according to their probable function in the cell based on information obtained from studies of other cellular systems. Proteins were identified that are involved in the cytoskeleton, the cellular membrane, and cell growth and division. In addition, several enzymes and two proteins of uncertain function were identified. As discussed in the following paragraphs, the nature of the identified proteins suggests that the stimulation of endothelin receptors by ET-1 unleashes rapid responses in a wide and complex set of cellular processes.

Cell Growth and Division. The identified proteins that are likely to influence the cell cycle are protein phosphatase 2A, cdc25A, and procaspase-2. ET-1 has been reported to promote cell proliferation through both ETA and ETB receptors (*27*, *28*). One would therefore expect that the modifications observed for these proteins would have a positive effect on cell cycle progression.

Protein phosphatase 2A (serine/threonine protein phosphatase 2A) is a phosphatase widely expressed in eukaryotic cells. Previous studies have shown it to be present in fibroblast cells (e.g., murine 10T1/2 fibroblasts (*29*)). There is good evidence for phosphorylation at a conserved tyrosine residue in the C-terminus, and this has been shown to decrease phosphatase activity (*30*). Decreased phosphatase activity has also been associated with phosphorylation at a threonine residue, although this has only been observed in vitro (*31*). In addition, work in rat brain and human erythrocytes has shown that the B″ (δ subunit) is phosphorylated at Ser^{60}, Ser^{75}, and Ser^{573} by cAMP-dependent protein kinase and that this phosphorylation activates PP2A (*32*, *33*). PP2A was identified here as being serine phosphorylated after stimulation with ET-1 (Table 1), but at sites Ser^{27} and Ser^{31}, it seems not to have previously been reported.

PP2A is considered overall to be a negative regulator of the cell cycle. Inhibition of PP2A using okadaic acid leads to increased cell entry into the M-phase (*34*), and tyrosine phosphorylation of PP2A on stimulation of the cell with growth factors is believed to transiently deactivate PP2A, allowing a progression to cell division (*30*). At this stage in the cell cycle, dephosphorylation of cdc25 by PP2A inhibits entry into the M-phase (*35*, *36*). This information would suggest that ET-1, by causing serine phosphorylation of PP2A and hence increasing activation, could act against cell cycle progression. However, it has also been shown that earlier in the cell cycle, PP2A acts as a positive regulator, promoting the transition from G1 to the S-phase (*37*). Since the other proteins identified here also promote progression from G1 to the S-phase, it seems that transient serine phosphorylation of PP2A promotes cell cycle progression in response to ET-1 stimulation. Reversal of this phosphorylation, or further phosphorylation at tyrosine and/or threonine, may then occur when progression from G2 to the M-phase is required.

Cdc25A (also known as the M-phase inducer phosphatase 1) is a dual-specificity phosphatase. Cdc25A acts at two important points in the cell cycle: the transition from G2 into the M-phase (*38*) and the transition from G1 to the S-phase (*39*). It is widely expressed across cell types and is stabilized by serine phosphorylation (*38*). This increases the levels of cdc25A and pushes forward the cell cycle by increasing the capacity to activate cyclin B-Cdk1. It has been shown to have oncogenic potential (*40*). The sites for serine phosphorylation were not determined in this earlier work and might be different from the sites we have identified. Cdc25A is known to dephosphorylate cyclin-dependent kinase 2 during the cell cycle, and its absence results in the persistence of the inactive, phosphorylated form of Cdk2. This results in cell cycle arrest, which is a protective mechanism after DNA damage by radiation.

In addition, cdc25A can dephosphorylate the EGF receptor (EGFR), with which ET-1 stimulation is known to interact to promote the G1 to S-phase transition (*41*, *42*). The in vivo effect of this dephosphorylation is not clear. The mitogenic action of ET-1 is thought to be at least in part due to the phosphorylation of EGFR, which increases its tyrosine kinase activity. This would suggest that increasing levels of cdc25A due to serine phosphorylation would diminish EGFR activity. However, it has also been observed that the hyperphosphorylation of EGFR that occurs when cdc25A is inhibited is associated with the activation of MAPK pathways that induce cell cycle arrest or cell death (*43*). Given the uncertainties in the earlier work about which cdc25A sites are phosphorylated on serine, interactions between EGF receptor and cdc25A are not necessarily in conflict with the promotion of cell cycle progression.

We observed threonine phosphorylation of cdc25A on stimulation with ET-1, which seems not to have been

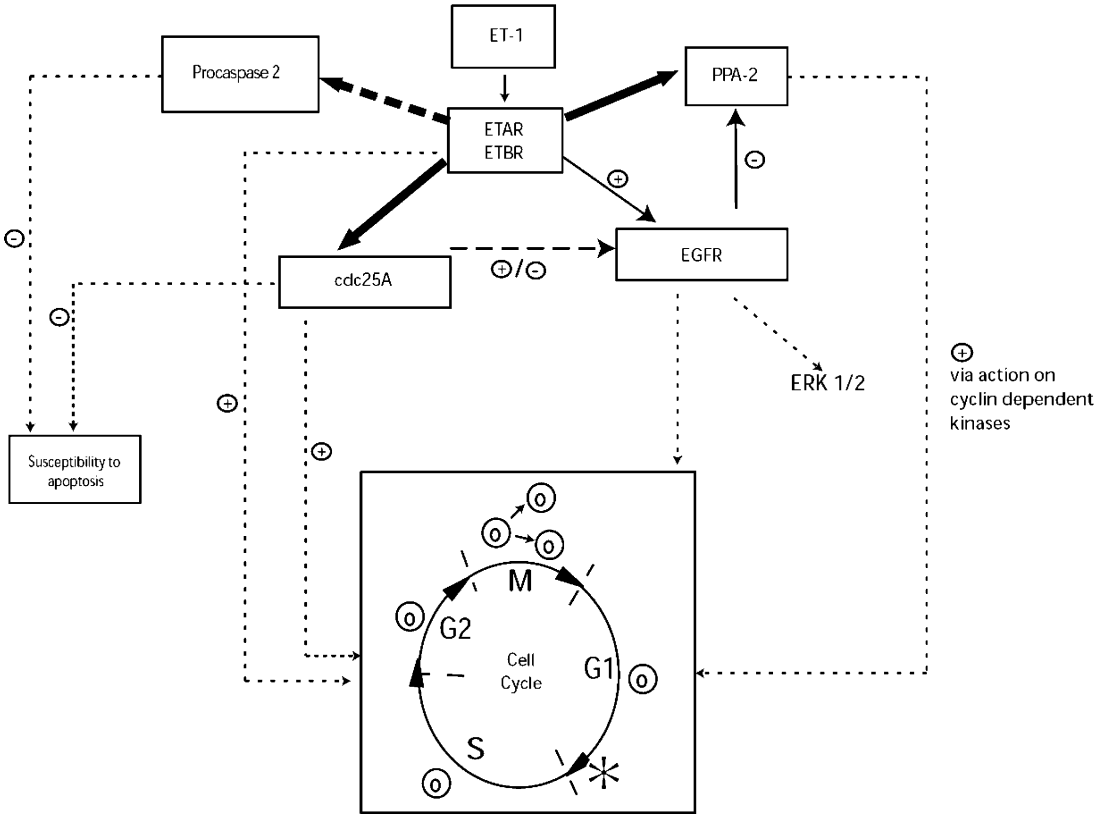

FIGURE 5: Possible mechanisms by which ET-1 could promote the G1/S-phase transition in the cell cycle. The phosphorylation of the proteins procaspase-2, PP2A, and cdc25A was identified as being modified on stimulation of the cells with ET-1. ± indicates the action of the protein at the start of the arrow on the activity of the protein or process at the end. Arrows denote the mechanism. Bold arrows represent the modification seen in our experiments (solid = phosphorylation, dashed = dephosphorylation). Dotted arrows are used where an effect has been recorded in the literature, but direct phosphorylation has not (yet) been implicated as the mechanism. Other arrows represent phosphorylation (solid) and dephosphorylation (dashed) that has been documented in the literature. The action of ET-1 to promote the G1/S-phase transition may be a combination of many pathways, including those suggested here, and is represented by a broken black arrow.

reported previously. This could promote the stability of cdc25A, which would lead to increased levels and to the promotion of cell cycle progression at both the G1/S-phase and the G2/M-phase. This would be synergistic with the effect of the serine phosphorylation of PP2A at the G1/S-phase transition. The action of increased levels of cdc25A on EGFR could be either synergistic or in opposition.

The final modified protein suggested as a cell cycle regulatory protein is procaspase-2 (caspase 2 precursor protein). Caspases are a group of widely expressed proteins involved in the execution of programmed cell death. A recent paper has suggested an important role for caspase-2 in the initiation of apoptosis (*44*). We observed dephosphorylation at serine for procaspase-2, the inactive precursor for caspase-2. There is evidence from other cell types that phosphorylation of procaspases occurs (unpublished work cited in ref *45*), but the functional significance of this is not clear. If dephosphorylation of procaspases promotes conversion to caspase-2, the effect of ET-1 stimulation would be pro-apoptotic, which it is not. We therefore speculate that the dephosphorylation of procaspase-2 maintains procaspase-2 in its inactive form, thereby promoting cell survival.

Overall, we have identified three proteins known to be involved in the regulation of the cell cycle that are modified on stimulation of human lung fibroblasts with ET-1. The observed modifications could, in each case, be associated with progression from G1 to the S-phase. The combination of these three separate (or interlinked) mechanisms would contribute to a powerful effect of endothelin on the progression of the cell cycle at this stage, which is consistent with reports that ET-1 does indeed act to promote the G1/S-phase transition. Figure 5 shows diagrammatically how these three proteins might link with ET-1 stimulation and cell cycle progression.

In a previous study of new protein synthesis induced by ET-1, we have shown changes in the synthesis of transcription factors such as Sox 5 (*46*). In this context, it is interesting to note that dephosphorylation at Ser occurs for the Kruppel-related zinc-finger protein 2 (HRK-2). The Kruppel-related zinc finger proteins are a group of DNA binding proteins that are involved in transcription and constitute components for transcription factors such as SP1 (*47*). This is an indication that changes in protein synthesis are also initiated at a very early stage following stimulation with ET-1.

Membrane Proteins. Two proteins relating to ion channels were identified as having been phosphorylated following endothelin stimulation: Kv3.4, a voltage-gated potassium channel, and stomatin (erythrocyte band 7), a membrane protein believed to be a regulator of ion channel function.

The identification of Kv3.4 from fibroblasts was initially somewhat surprising. However, voltage-gated potassium channels have been shown to be present in fibroblasts (*48*, *49*). Furthermore, phosphorylation is known to regulate the activity of several voltage-gated ion channels including Kv3.4

channels (*50*, *51*). Phosphorylation at several serine residues has been shown to modulate the inactivation time of the channel by different degrees according to the site. In some cases, inactivation time was increased, thus reducing the current, whereas in others the inactivation time was reduced. This is thought to be due to structural changes in the inactivation domain of the channel, which were observed by NMR spectroscopy when specific residues were phosphorylated (*51*). Physiologically, this phosphorylation is thought to result from activation of PKC, and ET-1 is able to activate PKC via the IP3/DAG pathway. The phosphorylation that we observed was on threonine rather than serine residues but suggests that ET-1-induced phosphorylation may have a regulatory role for Kv3.4 channels. This is further supported by the observation that ET-1 inhibits currents through voltage-gated potassium channels in pulmonary artery myocytes, probably by a PKC dependent mechanism (*52*). PKC can phosphorylate both serine and threonine residues, so it may be that threonine phosphorylation as a consequence of ET-1 stimulation also has a part to play in the modulation of channel activity.

Stomatin is a membrane protein named for its absence from the red blood cells of patients with Overhydrated Hereditary Stomatocytosis. These patients show an ion leak from their red cells, and it has been proposed that stomatin acts as a regulator of cation channels in the red cell (*53*). More recently, other functions have been proposed (e.g., as a scaffolding protein). This might unify its role across cell types, but overall, the function of stomatin remains unclear (*54*). The presence of stomatin in fibroblasts seems not to have been previously reported, but it is not limited to the red cell, and similar proteins are seen across many organisms, including plants (*55*). Stomatin is known to be phosphorylated at Ser^9 (*56*). We observed threonine phosphorylation of stomatin, but at present, it is not clear how this is involved in the cellular response to endothelin.

γ_2-Adaptin (adaptor-related protein complex 1, γ 2 subunit) is a protein homologous to γ-adaptin, which is part of the AP-1 adapter complex and is known to be expressed in the lung (*57*). AP-1 is involved in protein sorting at the transgolgi network of the cell, while its well-known partner AP-2 is localized to the membrane and takes part in clathrin-coated pit endocytosis. While γ_2-adaptin is homologous to γ-adaptin, it does not colocalize with it in the cell, nor does it interact with the same set of proteins (*57*, *58*). This suggests a function for γ_2-adaptin distinct from the AP-1 γ-adaptin, but this function is not clear. Phosphorylation of the α and β subunits of the AP-1 complex has been reported and is believed to control the interaction between clathrin and adapter complex, but phosphorylation of the γ subunit was not reported (*59*). Overall, it is plausible to suggest that ET-1 stimulation has an influence on intracellular trafficking. The receptor itself is internalized on stimulation, at least in part by the clathrin-coated pit mechanism (*60*, *61*), and we have previously shown that ET-1 stimulation of human lung fibroblasts leads to changes in protein synthesis for Rab3 and two isoforms of Rab14 (*46*).

Cytoskeletal Proteins. The proteins actin, vimentin, and T-plastin are components of the cytoskeleton. Serine phosphorylation of T-plastin and both tyrosine and threonine dephosphorylation of actin and vimentin were observed on stimulation with ET-1. Actin and vimentin are two of the most abundant proteins in fibroblast cells. These two proteins were included despite the fact that they appear in both stimulated and unstimulated gels because there was a major change in the level of tyrosine and threonine phosphorylation between the stimulated and the unstimulated gels (Figures 1 and 2). This change was not observed in the anti-phosphoserine gel (Figure 3), suggesting that there was no overall decrease in the levels of these proteins.

The functional significance of dephosphorylation of cytoskeletal components is not clear. Cytoskeletal rearrangement is a consequence of many stimuli including the cell cycle and external stressors. There is some evidence that dephosphorylation may be associated with growth and survival. In plants, the dephosphorylation of actin is associated with germination (*62*, *63*). Increasing levels of tyrosine phosphorylation are associated with the breakdown of actin filaments, which is reversible as tyrosine phosphorylation is decreased (*64*). Phosphorylation of vimentin is also associated with increasing disorganization of the cytoskeleton via disassembly of intermediate filaments (*65*). It has been shown that dephosphorylation of vimentin can be accomplished by PP2A, a phosphatase that we observed to be phosphorylated (upregulated) on ET-1 stimulation. In astrocytes, process formation is inhibited by ET-1 and requires decreased tyrosine phosphorylation of the actin-associated proteins paxillin and focal adhesion kinase (FAK) (*66*). ET-1 has been shown to increase the tyrosine phosphorylation of FAK and paxillin (*67*). Application of ET-1 to Schwann cells is associated with cell spreading and shorter processes, which is considered a proliferative phenotype (*68*). Although the functional significance is not yet clear, it seems likely that the dephosphorylation of actin and vimentin observed here for ET-1 stimulation (Figures 1 and 2) is associated with increasing cytoskeletal organization or at least stabilization of actin and intermediate filaments.

Plastins (or fimbrins) are a family of actin-bundling proteins with different tissue distributions. L-Plastin is expressed in leukocytes. T-Plastin is expressed in almost all eukaryotic cells other than leukocytes. T-Plastin was seen to be serine phosphorylated on cell stimulation with ET-1 (Table 1). L-Plastin has been shown to be serine phosphorylated, probably by PKC (*69*), although phosphorylation of T-plastin was not observed. It seems likely that the observed phosphorylation of T-plastin on stimulation with ET-1 occurs as part of cytoskeletal changes since T-plastin has been shown to be involved in the organization of actin structures (*70*).

Enzymes. Cytochrome P450 51 (CYP51) is a member of a large family of mono-oxygenase enzymes that are involved in many aspects of cell metabolism. CYP51 mRNA is expressed in most tissues, including the lung (*71*). CYP51 is highly conserved between species and is present in all biological kingdoms. The main known function of CYP51 is in the biosynthesis of cholesterol. Its ubiquitous expression and the structure of its gene suggest a housekeeping role for CYP51 (*72*). Other members of the CYP family (e.g., CYP2E1, CYP2B1) are down-regulated by serine phosphorylation, which occurs by the activation of PKA in response to an increase in cAMP concentration (*73*, *74*). ET-1 stimulation can cause also increased cAMP concentrations, and a similar mechanism might account for the serine and threonine phosphorylation observed for CYP51. However,

there is no information available on whether phosphorylation modulates the activity of CYP51, and since serine phosphorylation by PKA is much more common than threonine phosphorylation, it may be that other kinases are involved (*75*). Nonetheless, it is interesting to note that ET-1 might also lead to modulation of cellular properties by this type of pathway.

GFAT-1 (glutamine:fructose-6-phosphoate-amidotransferase) is an enzyme involved in glucose metabolism. It is expressed in fibroblasts, although this may not be at detectable levels when they are quiescent (*76*). It has been shown to be serine phosphorylated at Ser^{205} and Ser^{235} by PKA, which is consistent with our finding of serine phosphorylation. Phosphorylation at Ser205 deactivates the enzyme, pushing the pathway towards energy production rather than synthetic functions (*77*). How this would be involved in the response to endothelin stimulation is not clear.

Other Proteins. Phosphorylation at Thr was observed for angiopoietin-related protein 2. This is a secreted protein that has been shown to induce sprouting of endothelial cells via autocrine and paracrine actions. It is expressed in many tissues, although there seems to be no report of expression in the lung (*78*). Endothelin is known to be a promoter of angiogenesis by direct and indirect stimulation of endothelial cells (*79*), and there is evidence that it may be involved in tumor vascularization. An indirect action via VEGF has been shown (*22*). Regulation of the secretion of angiopoietin-related protein 2 might be another mechanism by which ET-1 could influence angiogenesis.

The only specific work on mosaic protein LGN reported its identification and interaction with the α2 subunit of the inhibitory G-protein G_i (*80*). It has been shown that ET-1 stimulation can lead to the interaction of both endothelin receptors with G_i proteins (*81*). Thus, serine phosphorylation of mosaic protein LGN might be involved in the regulation of the classical pathways for G-protein-coupled receptors such as the endothelin receptors, but the significance of the phosphorylation of this protein is at present unclear.

At present, the possible mitogenic effects of ET-1 stimulation are most amenable to interpretation and have led us to propose a new mechanism for the mitogenic effects of ET-1 (Figure 5). Overall, the set of proteins that show rapid modulation of phosphorylation as a consequence of stimulation of human lung fibroblasts by ET-1 (Table 1) points to a rich and varied engagement of many cellular pathways. This emphasizes the enormous potential of proteomics methods to concurrently monitor many diverse responses. Indeed, the small number of proteins that have so far been identified by MS already point to many unsuspected ramifications of ET-1 signaling. It should be noted that we have also recently demonstrated that the ETA and ETB receptors from human lung fibroblasts themselves are subject to very complex patterns of phosphorylation and that these patterns are rapidly changed following stimulation (*24*).

It is clear that the new methods we have developed for the extraction and fractionation of the phosphoproteome have resulted in a vast increase in the number of phosphorylated proteins that can be monitored (Figures 1−4). To fully exploit this advance, a number of other technical advances are desirable and are in progress in our labs. This includes an instrument for automated fractionation of cellular phosphoproteins, which is particularly helpful in isolating low abundance phosphoproteins that are phosphorylated exclusively on only one Ser, Thr, or Tyr (*82*). Also important is the development of multiphoton-detection (MPD) imaging methods, which allow cellular proteins obtained under two different conditions (e.g., stimulated or not, to be compared on a single 2-D gel (*83*)). This obviates difficulties in identifying and comparing spots on two different gels. Finally, many of the proteins shown in Figures 1−4 show partial changes in the amounts of a given phosphorylated species. It will be desirable, and perhaps essential, to quantitate these changes to interpret their cellular relevance. For example, it is somewhat surprising that PP2A and cdc25A appeared to show on−off behavior in the present experiments. Given that both of these proteins are known to be multiply phosphorylated, it seems likely that it is particular phosphorylation isoforms of these proteins that have been detected in the present experiments. To verify this and to detect conversion to other isoforms, accurate quantization may be required. Because it detects emission of multiple photons/particles in single-decay events of appropriate types of radionuclei at radiation levels far below the background, MPD imaging provides an efficient means to obtain highly accurate, linear, and quantitative measurements of protein amounts over more than 7 orders of magnitude ranging upward from low zeptomole levels (*83*). As these new tools come on-line, it should be possible to achieve another dramatic increase in the ability of proteomics methods to monitor cellular function via the observation of changes in the phosphoproteome.

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