Systematic Analysis of the Epidermal Growth Factor Receptor by Mass Spectrometry Reveals Stimulation-dependent Multisite Phosphorylation*

Elisabetta Boeri Erba‡§¶, Elena Bergatto§, Sara Cabodi§, Lorenzo Silengo§, Guido Tarone§, Paola Defilippi§∥, and Ole N. Jensen‡**

Multisite phosphorylation of proteins is a general mechanism for modulation of protein function and molecular interactions. Definition of phosphorylation sites and elucidation of the functional interplay between multiple phosphorylated residues in proteins are, however, a major analytical challenge in current molecular cell biology and proteomic research. In the present study, we used mass spectrometry to determine the major phosphorylated residues of the human epidermal growth factor (EGF) receptor at various well defined cellular conditions. Activation of EGF receptor was achieved by several types of stimulation, i.e. by sodium pervanadate, EGF, and integrin-dependent adhesion. The contribution of cell-matrix adhesion was also determined by activating the EGF receptor by EGF in cells kept in suspension. We developed an analytical strategy that combined miniaturized sample preparation techniques and MALDI tandem mass spectrometry and determined a total of nine phosphorylation sites in the EGF receptor. We discovered one novel phosphorylation site (Ser967) and revealed constitutive phosphorylation of Thr669, Ser967, Ser1002, and Tyr1045 and stimulation-dependent differential phosphorylation of Tyr1068, Tyr1086, Ser1142, Tyr1148, and Tyr1173. The EGF receptor was purified from HeLa cells or ECV304 cells by immunoprecipitation and SDS-PAGE and then digested with trypsin. Phosphopeptides in the range of 0.8–3.7 kDa were recovered by combinations of IMAC, perfusion chromatography, and graphite powder chromatography and subsequently detected and sequenced by MALDI quadrupole time-of-flight tandem mass spectrometry. Two phosphorylation sites were detected in the peptide 1137GSHQIS-LDNPDYQQDFFPK1155; however, only Tyr1148 was phosphorylated upon EGF treatment; in contrast Ser1142 was only phosphorylated by integrin-dependent adhesion in the absence of EGF treatment, suggesting differential phosphorylation of this region by distinct stimuli. This MALDI MS/MS-based analytical approach demonstrates the feasibility of systematic analysis of signaling molecules by mass spectrometry and provides new insights into the dynamics of receptor signaling processes. Molecular & Cellular Proteomics 4:1107–1121, 2005.

Phosphorylation can alter protein structure and function and thereby modulate and control intrinsic biological activity, subcellular location, stability, and interaction with other proteins. Multisite phosphorylation, i.e. the phosphorylation of protein at multiple specific amino acid residues, encodes a variety of biological functions. In growth factor receptors multisite phosphorylation is a mechanism for recruitment of adapter, scaffolding, and signaling molecules to the plasma membrane for the propagation of external stimulatory cues into the cytoplasm and the nucleus of the cell.

The epidermal growth factor receptor (EGF1 receptor) is a 170-kDa protein receptor tyrosine kinase that, in response to ligand binding, is activated and mediates cell proliferation and cell migration (1). Mutations of EGF receptor sites and its overexpression are implicated in a variety of cancers, including mammary carcinomas, squamous carcinomas, and glioblastomas. The EGF receptor is composed of a large extracellular domain that contains the ligand-binding site, a single hydrophobic transmembrane region, and an intracellular domain. This intracellular domain contains the tyrosine kinase activity and a carboxyl-terminal region that is characterized by the presence of several important tyrosine residues. Upon receptor activation, these tyrosine residues are phosphorylated and act as docking sites for Src homology 2 domains of target molecules. Five autophosphorylation sites have so far been identified in the EGF receptor; all of them are clustered at the extreme carboxyl-terminal 194 amino acids. Among these sites, Tyr1068, Tyr1148, and Tyr1173 are major sites.

From the ‡Protein Research Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark and the §Department of Genetics, Biology and Biochemistry, Medical School, University of Torino, Via Santena, 5/bis 10126 Torino, Italy
Received, March 11, 2005, and in revised form, May 10, 2005
Published, MCP Papers in Press, May 18, 2005, DOI 10.1074/mcp.M500070-MCP200

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
This paper is available on line at http://www.mcponline.org
whereas Tyr992 and Tyr1086 are minor sites (2).

In addition to the canonical activation by its soluble ligands, EGF receptor responds to a host of signals outside the ligand family; EGF receptor transactivation has been detected by G-protein-coupled receptor agonists, phorbol esters, cytokines, estrogen, and cell stress signals (3). These data expand the traditional view of highly specific receptor-ligand interactions, suggesting a wealth of signals impinging on the EGF receptor. It is well known that integrins provide anchorage-dependent signals required for entry into S phase of the cell cycle in response to growth factors (4). Recent results show that the EGF receptor can also be transactivated by integrin-mediated adhesion and involved in cell survival and in actin cytoskeleton reorganization (5–7). In addition integrin-mediated adhesion cooperates with soluble ligand EGF in activation of EGF receptor downstream signaling (8).

Because of its biological significance and physiochemical properties the EGF receptor presents a highly interesting and relevant model for the development of new analytical techniques for investigations of multisite phosphorylation in proteins. To further evaluate the cross-talk between cell-matrix adhesion and EGF in EGF receptor phosphorylation we decided to compare phosphorylation of specific sites when EGF receptor is activated by soluble ligand EGF in adherent cells versus cells kept in suspension. We also wanted to analyze phosphorylation of the EGF receptor upon activation by integrin-dependent cell-matrix interaction.

Mass spectrometry is widely used for studies of protein phosphorylation (9–11). ESI and MALDI mass spectrometry are both amendable to phosphoprotein analysis, but most work to date has been performed using LC-MS/MS where peptide separation is combined with on-line ESI tandem mass spectrometry for automated peptide sequencing. MALDI mass spectrometry is a simple, sensitive, and robust technology for peptide analysis, but it has lacked practical capabilities and sensitivity for peptide sequencing. However, a range of MALDI tandem mass spectrometry instruments have been developed in recent years, including TOF-TOF (12), Q-TOF (13), and ion trap analyzers (14). We have previously demonstrated that MALDI-triquadropole TOF tandem mass spectrometry allows phosphopeptide sequencing and determination of phosphorylation sites (15). In addition, we have optimized sample preparation methods to enhance phosphopeptide recovery and detection by MALDI MS by using a combination of 0.8% phosphoric acid and 2,5-dihydroxybenzoic acid (DHB) as matrix (16, 17). This MALDI matrix is compatible with phosphopeptide enrichment by IMAC (17, 18) and graphite powder (19) and with peptide concentration/desalting by solid phase extraction by POROS R1, R2, or R3 resin (20). The capability of MALDI MS/MS for sequencing of large (>3-kDa) multiply phosphorylated peptides (16, 17) is an advantage over automated LC-MS/MS set-ups as the latter methods may fail to detect or efficiently sequence such species (21).

The aim of the present study was to develop a sensitive and robust analytical strategy for mapping of the major EGF receptor phosphorylation sites and applying this technology to study EGF receptor phosphorylation upon various stimuli. Multidimensional off-line separation methods in combination with MALDI MS and MS/MS analysis allowed us to achieve these goals. Serial application of miniaturized sample preparation techniques combined with MALDI quadrupole time-of-flight tandem mass spectrometry allowed us to determine nine phosphorylated amino acid residues in the EGF receptor, including several differentially phosphorylated residues and one novel phosphorylation site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) and FCS were from Invitrogen. mAbs HB-8509 and HB-8508 to the EGF receptor and mAb L230 to the αv integrin subunit were purchased from American Type Culture Collection (ATCC) and purified on protein A-Sepharose columns that were from GE Healthcare. mAbs HB-8509 and HB-8508 were bound to CNBr-activated Sepharose beads at a concentration of 10 mg/ml.

Sodium orthovanadate (Na3VO4), EDTA, Fe(II) chloride (FeCl2), ammonium hydrogen carbonate (NH4HCO3), dithiothreitol, iodoacetamide, graphite powder, and human recombinant EGF were obtained from Sigma. 1% Nonidet P-40 was from Roche Applied Science. Modified porcine trypsin was from Promega (Madison, WI). GE-Loader tips were purchased from Eppendorf (Hamburg, Germany). ACN (HPLC grade) was from Fisher Scientific; ortho-phosphoric acid (85%) and hydrogen peroxide (30%) were from J. T. Baker Inc. Formic acid (FA) was purchased from Merck KGaA (Darmstadt, Germany), and acetic acid was from Fluka (Buchs, Switzerland). All chemicals used were ACS or HPLC grade. All water used was obtained from a Milli-Q system (Millipore, Bedford, MA). POROS R1, POROS 10 R2, and POROS Oligo R3 reverse phase chromatography medium was from PerSeptive Biosystems. Ni(II)-nitrilotriacetic acid (NTA)-silica (16–24-mm particle size) was from Qiagen (Hilden, Germany). Alkaline phosphatase was from Roche Applied Science (20 units/μl). DHB matrix was purchased from Aldrich.

**Cell Lines and EGF Receptor Purification**—Human cell lines HeLa and ECV304 were purchased from ATCC and grown in standard conditions with DMEM in the presence of 10% FCS. A Coomassie-stainable band of EGF receptor could be obtained by SDS-PAGE from 2.5 dishes (15-cm diameter) of HeLa cells. In the case of ECV304 cells, four dishes were used to obtain EGF receptor for each of the experimental conditions.

HeLa cells were grown to confluence, starved for 18 h, and then treated with a solution of sodium pervanadate. 100 mM of Na3VO4 (sodium vanadate) was prepared and mixed in equal amount with 100 mM H2O2. The generated 50 mM sodium pervanadate solution was used within 5 min to minimize decomposition of the vanadate-hydrogen peroxide complex. Cells were treated at a final concentration of 1 mM for 30 min at 37°C. ECV304 cells grown to confluence were serum-deprived in DMEM for 24 h and treated in different conditions. Cells were detached with 10 mM EDTA in PBS, washed, and plated for 30 min on αv integrin subunit antibody-coated dishes (10 μg/ml) in the presence or absence of 50 ng/ml recombinant EGF. EGF was also given for 30 min to cells kept in suspension.

At the end of the experiment, cells were washed with a PBS buffer containing 5 mM EDTA, 10 mM NaF, 10 mM Na3PO4, and 1 mM Na3VO4 and detergent-extracted in lysis buffer containing 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA, 10 mM NaF, 10 mM Na3PO4, 0.4 mM Na3VO4, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 unit/ml aprotinin). Cell
lysates were centrifuged at 13,000 × g for 10 min, and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method. For immunoprecipitation experiments, 4–6 mg of protein extracts were loaded on the column of mAbs HB-8509 and HB-8508 (purchased from ATCC) for 4 h at 4 °C, washed, and resolved by 6% SDS-PAGE. Proteins were stained using colloidal Coomassie G250 solution (Acros Organic, Geel, Belgium).

In-gel Digestion of EGF Receptor Separated by SDS-PAGE—After SDS-PAGE, EGF receptor-containing bands were cut from the gel and rinsed followed by in-gel reduction, S-carbamidomethylation, and digestion with 12.5 ng/μl trypsin overnight at 37 °C; the peptides were extracted by three changes of 5% FA in 50% ACN (20 min for each change) at room temperature and dried down in a SpeedVac (22).

Phosphopeptide Purification by Nanoscale Fe(III)-IMAC and Fractionation by POROS R1, R2, and OLIGO R3 and Graphite Powder—Before the mass spectrometric analysis the peptide mixtures were fractionated by custom made desalting/concentration columns used in series: the flow-through from one column was collected in the following one.

A slurry of Fe(III)-loaded NTA-silica resin was prepared by first resuspending the resin-Ni(II)-NTA in water (~1 mg/ml), and then it was treated, in turn, with 0.1 M EDTA, 0.2 M acetic acid, and 0.1 M FeCl3 in 0.2 M acetic acid (1:1, v/v) before two equilibration steps with 0.2 M acetic acid. This ready-for-use slurry of Fe(III)-IMAC resin in 0.2 M acetic acid was stored at 4 °C.

The miniaturized columns were prepared in-house according to Gobom et al. (20). A long, narrow pipette tip was carefully flattened near the end of the outlet, and a suspension of chromatography medium in 0.2 m acetic acid for Fe(III)-IMAC resin or pure ACN for POROS R1, R2, and OLIGO R3 and graphite material was deposited in the pipette tip and gently pressed through to pack a column of 2–3 mm in length near the outlet. A plastic syringe was used to apply pressure to the liquid. The crude EGF receptor digestion mixture was loaded onto Fe(III)-IMAC (18) POROS R1, R2, and OLIGO R3 (23) and graphite (24) columns.

Peptide samples were diluted to a final volume of 30–40 μl using 0.1 m acetic acid and loaded very slowly (0.5–2 μl/min) onto the Fe(III)-IMAC column. The Fe(III)-IMAC column was equilibrated with 0.1 m acetic acid and washed with 30% ACN in 0.1 m acetic acid.

The flow-through of the Fe(III)-IMAC column was collected, acidified with 20 μl of 5% FA, and subjected to sequential solid phase extraction on R1, R2, and R3 POROS resin and graphite powder columns. The samples were eluted with 1 μl of 20 mg/ml matrix solution containing DHB matrix dissolved in 50% ACN, 0.8% ortho-phosphoric acid. The matrix/analyte eluate was spotted as a series of droplets on the MALDI target.

Enzymatic Dephosphorylation of Phosphopeptides—The peptides were eluted from IMAC columns by 10 μl of pH 10.5 solvent and split into aliquots. One aliquot was transferred to a POROS R2 column and diluted in 15 μl of dephosphorylation buffer (1 unit of alkaline phosphatase in 20 μl of 100 mM NH4HCO3). After incubation for 45 min at 37 °C it was acidified by addition of 5% formic acid. The other aliquot was acidified and loaded directly into a POROS R2 column (18).

The samples were eluted from the column using 1 μl of 20 mg/ml matrix solution prepared with DHB dissolved in 50% ACN, 0.8% ortho-phosphoric acid. The matrix/analyte eluate was spotted as a series of droplets on the MALDI target.

Peptide Mass Mapping by MALDI-TOF MS and Peptide Sequencing by MALDI-Q-TOF MS/MS—MALDI mass spectra were recorded on a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) operating in the positive ion delayed extraction reflector mode. Ions were generated by irradiation of analyte/matrix deposits by a nitrogen laser at 337 nm and analyzed with an accelerating voltage of 20 kV. Each MALDI-TOF spectrum was generated by accumulating data corresponding to 300 laser shots. Mass calibration in the range m/z 800–4000 was performed by using a bovine β-lactoglobulin tryptic peptide mixture peak. Postacquisition internal calibration using theoretical masses of tryptic peptides from the analyzed proteins was applied to increase mass accuracy. MALDI tandem mass spectra were recorded on a MALDI-Q-TOF tandem mass spectrometer (Ultima HT, Waters/Micromass, Manchester, UK) equipped with a nitrogen laser (λ = 337 nm). Poly(ethylene glycol) was used for instrument mass calibration in the m/z 400–4000 range. The collision energy applied to fragment singly protonated molecular peptide ions was in the range of 60–180 eV. To reduce the sample consumption only 300 laser shots were used to acquire MALDI mass spectra using the Q-TOF instrument, whereas more shots were used to obtain MS/MS spectra for sequencing of phosphopeptides.

RESULTS AND DISCUSSION

Analytical Strategy for Identification of EGF Receptor Phosphorylation Sites—The analytical strategy used in this study is depicted in Fig. 1. First the EGF receptor was immunoprecipitated from a human cell lysate and isolated by SDS-PAGE. Next the protein was in-gel digested by trypsin, and the resultant peptides were extracted. We then applied a multidimensional separation scheme using sequential custom made miniaturized columns for phosphopeptide enrichment combined with MALDI MS and MS/MS analysis for phosphopeptide detection and sequencing.

In our first experiments, we set out to analyze EGF receptor that was recovered by immunoprecipitation of lysates prepared from a sodium pervanadate-treated human HeLa epidermal carcinoma cell culture. Pervanadate is a general inhibitor of protein tyrosine phosphatases (PTPases) and was used to increase the abundance of phosphorylated EGF receptor. In subsequent experiments we systematically studied adhesion-dependent phosphorylation of the EGF receptor from ECV304 cells grown in suspension with stimulation by soluble EGF or under adhesion conditions with and without EGF treatment (see below).

The EGF receptor was immunoprecipitated from cell extracts using a resin containing two specific monoclonal antibodies against the EGF receptor. These antibodies recover all phosphorylated forms of the receptor because they recognize and bind to epitopes in the extracellular ligand binding region. Immunoprecipitated EGF receptor was isolated by SDS-PAGE, and EGF receptor peptides were recovered after in-gel digestion using trypsin. To enhance the phosphopeptide detection efficiency, tryptic peptide mixtures derived from the digested EGF receptor were fractionated prior to mass spectrometry analysis. At the first stage, Fe(III)-IMAC, which exploits the high affinity of phosphate groups toward the chelated metal ion stationary phase Fe(III)-NTA was used (17, 18). The Fe(III)-IMAC column is expected to retain a majority of phosphopeptides, but to also recover those phosphopeptides that are not efficiently retained by IMAC and to minimize sample losses we combined this method with other miniaturized chromatographic techniques. Thus, the flow-through
(void volume) of the Fe(III)-IMAC column was collected and subjected to sequential reverse phase chromatography on POROS R1, R2, and R3 resin (20, 23) and graphite powder columns (24), respectively. These resins are increasingly hydrophobic, thereby retaining only subsets of peptides. The void volume of the R1 column was loaded onto the R2 column, the void volume of the R2 column was loaded onto the R3 column, and so forth with the R3 and graphite columns.

The graphite powder has a high capacity to bind small or hydrophilic (phospho)peptides. It is used as the last stage to take advantage of this selectivity as large and hydrophobic peptides will supposedly bind to the other resins (IMAC or POROS R1, R2, or R3). The binding of large peptides to graphite is very strong, and it is sometimes very difficult to recover such species from the graphite column.

In each step, retained peptides were eluted from the columns by using a small aliquot of DHB matrix solution with ortho-phosphoric acid and spotted directly onto the MALDI MS and MS/MS target (Fig. 1). Subsequently MALDI-TOF MS and MALDI-Q-TOF tandem mass spectrometry were utilized for phosphopeptide mapping and sequencing for determination and localization of the phosphorylated amino acid residues (15, 17). This protocol allows handling of subpicomole amounts of peptides and minimizes the sample losses by recovering a large portion of the tryptic peptides and phosphopeptides for MALDI MS and MS/MS analysis as described below.

Mapping of Phosphopeptides and Phosphorylation Sites in EGF Receptor by MALDI MS—MALDI-TOF MS peptide mass mapping and computational analysis confirmed that the protein recovered from the SDS-PAGE gel contained only the EGF receptor. Fe(III)-IMAC combined with MALDI MS and MALDI MS/MS allowed us to identify and sequence four singly phosphorylated peptides at $m/z$ 3719.50, 3478.58, 2315.99, and 2114.05 and one doubly phosphorylated peptide at $m/z$ 3558.54 (Table I and Fig. 2).

Phosphopeptide candidates were initially assigned by MALDI-TOF MS by their 79.96-Da mass increments per phosphate moiety relative to the unmodified peptides. To eliminate false positive assignments the peptides eluted from the Fe(III)-IMAC column were subjected to alkaline phosphatase dephosphorylation assays (17) (data not shown). By this procedure, the phosphorylated peptides exhibit a mass decrease of 79.96 Da per phosphate moiety, whereas nonphosphorylated peptides remain unchanged in the mass spectrum. The phosphopeptide candidates were then sequenced by MALDI-Q-TOF MS/MS (see below).

The Fe(III)-IMAC column flow-through, containing the unrecovered peptides, was collected and then passed over serial reverse phase type chromatographic columns in the order POROS R1, POROS R2, POROS R3, and finally a graphite powder resin (19). The order of the use of the different microcolumns was established to bind first the most hydrophobic and afterward the hydrophilic peptides. The R1 column led to the recovery and detection of an additional phosphopeptide at $m/z$ 2479.18. The R2 column recovered three phosphopeptides that had already been observed in the Fe(III)-IMAC column were subjected to alkaline phosphatase dephosphorylation assays (17) (data not shown). By this procedure, the phosphorylated peptides exhibit a mass decrease of 79.96 Da per phosphate moiety, whereas nonphosphorylated peptides remain unchanged in the mass spectrum. The phosphopeptide candidates were then sequenced by MALDI-Q-TOF MS/MS (see below).

The Fe(III)-IMAC column flow-through, containing the unrecovered peptides, was collected and then passed over serial reverse phase type chromatographic columns in the order POROS R1, POROS R2, POROS R3, and finally a graphite powder resin (19). The order of the use of the different microcolumns was established to bind first the most hydrophobic and afterward the hydrophilic peptides. The R1 column led to the recovery and detection of an additional phosphopeptide at $m/z$ 2479.18. The R2 column recovered three phosphopeptides that had already been observed in the Fe(III)-IMAC eluate (Table I). The R3 column bound a phosphopeptide at $m/z$ 1660.68. Finally the graphite powder column, which has been demonstrated to retain the most hydrophilic peptides, recovered an additional phosphopeptide at $m/z$ 1290.53.

In summary, eight phosphopeptides were detected in the EGF receptor after stimulation with sodium pervanadate and immunoprecipitation from human HeLa epidermal carcinoma cell culture. Five phosphopeptides were recovered by IMAC, whereas three additional phosphopeptides were retained by POROS R1 and R3 resins and graphite powder resin, respectively.
In addition to the sites that were experimentally determined by MALDI MS and MS/MS, we also tried to identify additional predicted tryptic phosphopeptides. We looked for tyrosine phosphorylation of residues 845, 891, and 920, three potential targets of c-Src kinase activity (26–28). The peptides 

\[837\text{LLG}---\text{GGK851} (m/z 1630.62), \quad 844\text{EYHAEGGK851} (m/z 1327.70)\] 

and 

\[844\text{EYH}---\text{PIK855} (m/z 1327.70)\]

that all include Tyr845 were present in the spectra obtained from the R2, R3, and graphite fractions, but the corresponding phosphorylated peptides were absent in these samples (data not shown). The known phosphorylation site at Tyr891 was contained in the tryptic peptides 

\[856\text{WMAL}---\text{SILEK905} (m/z 5770.86), \quad 1045\text{YSSDPTGALTEDSIDDTLPVEIYINQSPV3107} + \]

Finally, the flow-through from the R3 column was collected onto a graphite powder column, and an additional phosphopeptide at m/z 1290.53 was recovered. EGF receptor was immunoprecipitated from HeLa cells stimulated with pervanadate, a known inhibitor of tyrosine PTPase activity. EGFR, EGF receptor.

**TABLE I**

<table>
<thead>
<tr>
<th>Phosphopeptides derived from immunoprecipitated EGF receptor from HeLa cells stimulated with pervanadate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eight EGF receptor phosphopeptides were recovered by miniaturized chromatographic columns. Five phosphopeptides were recovered by IMAC. R1 column leads to the detection of an additional phosphopeptide with m/z 2479.18; the R2 column binds to three phosphopeptides already identified with the Fe(III)-IMAC column. In contrast the R3 column allows the binding of a phosphopeptide with m/z 1660.68. Finally the flow-through from the R3 column was collected onto a graphite powder column, and an additional phosphopeptide at m/z 1290.53 was recovered. EGF receptor was immunoprecipitated from HeLa cells stimulated with pervanadate, a known inhibitor of tyrosine PTPase activity. EGFR, EGF receptor.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>m/z</th>
<th>EGFR phosphopeptides detected after stimulation with sodium pervanadate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2114.05</td>
<td>666ELVEPLPSGEAPNQALLR861 + + +</td>
</tr>
<tr>
<td>1660.68</td>
<td>966MHLPSPDTSNFYR875 +</td>
</tr>
<tr>
<td>3719.5</td>
<td>977ALMDDEDDDDDDVDAEYLIPOQGFFSPSTSR1007 +</td>
</tr>
<tr>
<td>3558.54</td>
<td>1045YSSDPTGALTEDSIDDTLPVEIYINGSPV1075 +</td>
</tr>
<tr>
<td>3478.58</td>
<td>1137RPAGSVONPVPYHQPQLNPAPSR11097 +</td>
</tr>
<tr>
<td>2479.18</td>
<td>1166GSHQISLDNPDYQDFFPK1155 +</td>
</tr>
<tr>
<td>2315.99</td>
<td>1290.53</td>
</tr>
</tbody>
</table>

**Fig. 2.** EGF receptor phosphopeptide recovery by miniaturized chromatographic columns. EGF receptor was immunoprecipitated from cells stimulated with pervanadate. First the Fe(III)-IMAC column was applied to enrich phosphopeptides; afterward R1, R2, R3, and graphite columns were used. The order of the use of the various resins (R1 R2, R3, and graphite) was established to bind first the most hydrophobic and afterward the hydrophilic peptides. To reduce the sample consumption just 300 laser shots per spectrum were acquired.

In addition to the sites that were experimentally determined by MALDI MS and MS/MS, we also tried to identify additional predicted tryptic phosphopeptides. We looked for tyrosine phosphorylation of residues 845, 891, and 920, three potential targets of c-Src kinase activity (26–28). The peptides 

\[837\text{LLG}---\text{GGK851} (m/z 1630.62), \quad 844\text{EYHAEGGK851} (m/z 890.40), \quad \text{and} \quad 844\text{EYH}---\text{PIK855} (m/z 1327.70) \text{that all include} \]

Tyr845 were present in the spectra obtained from the R2, R3, and graphite fractions, but the corresponding phosphorylated peptides were absent in these samples (data not shown). The known phosphorylation site at Tyr891 was contained in the tryptic peptides 

\[856\text{WMAL}---\text{SILEK905} (m/z 5770.86), \quad 1045\text{YSSDPTGALTEDSIDDTLPVEIYINQSPV1075} + \]

and 

\[1137RPAGSVONPVPYHQPQLNPAPSR11097 + \]

Finally, the flow-through from the R3 column was collected onto a graphite powder column, and an additional phosphopeptide at m/z 1290.53 was recovered. EGF receptor was immunoprecipitated from HeLa cells stimulated with pervanadate, a known inhibitor of tyrosine PTPase activity. EGFR, EGF receptor.
Stimulation-dependent Multisite Phosphorylation of the EGF Receptor

866IYTHQ----SILEK905 (m/z 4534.22), and 866IYTHQ----EKGER905 (m/z 4876.38), but these were not detected by MALDI-TOF MS. The peptide 909LPQPPICTIDVYMIMVK925 that carries the potentially phosphorylated residue Tyr920 was detected only in the unphosphorylated form (data not shown). The fact that we did not detect these phosphopeptides does not rule out that they are actually present; however, we expect their extent of phosphorylation to be below the detection limit of our method.

In summary, these initial results demonstrate that it is necessary to apply more than one separation technology to recover tryptic phosphopeptides from large proteins. No single chromatographic material has proven to be adequate for binding all the phosphopeptides. We noted that by using a standard sample preparation method for MALDI peptide mass mapping based on the R2 column alone, only the phosphopeptides at m/z 2114.05, 3478.58, and 3719.50 were detected with low ion intensity.

The main advantages of using a series of complementary chromatographic columns for peptide separation is that it increases the probability for detection of phosphopeptides by MALDI MS as it fractionates the peptide sample and reduces the bias against detection of phosphopeptides (suppression effects). This concept of off-line separation prior to mass spectrometry is suitable for analysis of individual proteins but also for complex phosphopeptide mixtures (29, 30).

**Phosphopeptide Sequencing by MALDI MS/MS—** MALDI-Q-TOF MS/MS is a robust and sensitive technique for phosphopeptide sequencing, and we used this method to determine the exact sites of phosphorylation of the EGF receptor, which was isolated from human HeLa cells treated with pervanadate. The phosphopeptides corresponding to 976ALM-----TSR1007 (m/z 3719.50), 1045YSS----VPK1075 (m/z 3478.58 and 3558.54), and 1137GSH-----FPK1155 (m/z 2315.99) retained by IMAC columns were subjected to MS/MS analysis. The phosphopeptide 976–1007 was phosphorylated on Ser1002 as indicated by the y-ion series y5–y9 that localizes unambiguously the Ser(P)1002 and rules out the phosphorylation of the Tyr992 by the observation of y15 and y16 fragment ions (Fig. 3A). The interpretation of the MS/MS spectrum of phosphopeptides 1045–1075 locates the phosphorylation on Tyr1045 for the singly phosphorylated peptide (m/z 3478.58) and on Tyr1045 and Tyr1068 in the doubly phosphorylated peptide (m/z 3558.54) (supplemental data). By fragmentation of the phosphopeptide 1137–1155 (m/z 2315.99), the aminoterminval b-ion series indicates that the phosphorylated residue was Tyr1148 (data not shown, see also below).

**FIG. 3.** Phosphopeptide sequencing by MALDI QTOF MS/MS. A, MALDI-Q-TOF spectrum of phosphopeptide 976ALMDEEDMDDVDVADEYLPQQGFFSSPSTR1007 (m/z 3719.50) eluted from the IMAC column. *, ions containing dehydroalanine corresponding to Ser(P)1002. M*, oxidized Met. B, MALDI-Q-TOF MS/MS spectrum of phosphopeptide ELVEPLTSGEPNQALLR (m/z 2114.05) eluted from R1 column. *, ions containing dehydro-2-aminobutyric acid corresponding to Thr(P)669. For clarity, only the most relevant peaks are labeled. C, MALDI-Q-TOF spectrum of phosphopeptide 963MHLP-SPTDSFNYR975 (m/z 1644.68) (with oxidized Met, m/z 1660.66) eluted from R3 column. *, ions containing dehydroalanine corresponding to Ser(P)967. The presence of oxidized Met in position 963 induces a rearrangement with loss of methylsulfonic acid (64 Da) after the loss of phosphoric acid that is indicated in the spectrum as [M−H2PO4−CH3SOH+H]+. M*, oxidized Met; pS, phosphoserine; pT, phosphothreonine.
Similar analysis was performed on the phosphopeptide \(^{1076}\)RPAGSVQNPYHNQPLNPAPSR\(^{1097}\) (m/z 2479.18) recovered by the R1 column (Fig. 2). The results demonstrated that the phosphopeptide 1076–1097 was phosphorylated in Tyr\(^{1086}\) (supplemental data). The phosphopeptide \(^{663}\)ELVEPLTPS-GEAPNQALLR\(^{681}\) (m/z 2114.05) was bound to IMAC, R1, and R2 columns and was phosphorylated at Thr\(^{669}\) (Fig. 3B).

The phosphopeptide \(^{963}\)MHLPSPTDSNFYR\(^{975}\) (m/z 1660.68) was eluted from the R3 column (Fig. 2). MS/MS analysis (Fig. 3C) indicated that this peptide was phosphorylated on Ser\(^{967}\). The presence of oxidized Met in position 963 induces a rearrangement with loss of methylsulfonic acid (64 Da) after the loss of phosphoric acid as indicated in the spectrum as \([M - H_2PO_4 - CH_3SOH + H]^+\). To confirm the interpretation of the spectra, the peptides MHLPSPTDSNFYR, MHLPPPTDSNFYR, MHLPSPTDSNFYR, and MHLPSPTDSNFYR, where pS is phosphoserine and pT is phosphothreonine, were synthesized. MALDI-Q-TOF mass spectra of each synthetic peptide were acquired and compared with the spectrum of the phosphopeptide 963–975 eluted from R3 column, confirming the phosphorylation of Ser\(^{967}\) (31).

In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data). In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data). In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data). In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data). In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data). In summary, treatment of HeLa cells with pervanadate leads to the detection of phosphorylation on the residues Thr\(^{669}\), Ser\(^{967}\), Ser\(^{1002}\), Tyr\(^{1045}\), Tyr\(^{1068}\), Tyr\(^{1086}\), and Tyr\(^{1148}\), and Tyr\(^{1173}\) (supplemental data).
FIG. 5. MALDI MS analysis of phosphopeptides from the stimulated EGF receptor. A, IMAC column. EGF receptor was immunoprecipitated from ECV304 cells kept in different conditions: 1) in suspension upon EGF treatment (Susp + EGF), 2) in adhesion upon EGF treatment (Adh + EGF), and 3) in adhesion without EGF treatment (Adh). 0.5–1 μl of a 20-μl total peptide mixture was used for this MS experiment. B, R1 column. C, R2 column. D, R3 column. E, graphite column. pT, phosphothreonine; pY, phosphotyrosine; pS, phosphoserine.
Stimulation-dependent Multisite Phosphorylation of the EGF Receptor

C. R2

2114.05 (pT669)

Susp+EGF

Adh+EGF

2479.18 (pY1086)

D. R3

2114.05 (pT669)

1660.68 (pS967)

Susp+EGF

Adh+EGF

Adh

Fig. 5—continued
sitive enough to detect the major phosphorylated sites on the endogenous EGF receptor expressed on HeLa cells.

**EGF-dependent EGF Receptor Phosphorylation in Adherent Cells**—EGF is the physiological ligand of the EGF receptor, and therefore we set out to apply the MALDI MS/MS-based method to investigate the phosphorylation of the EGF receptor in response to 50 ng/ml EGF treatment in human ECV304 cells.

The ECV304 epithelial cell line was used in our previous studies to define some aspects of the molecular mechanisms regulating integrin-dependent EGF receptor phosphorylation (5, 6). On the basis of our previous data, we decided to extensively analyze EGF receptor phosphorylation in these cells because they express a good amount of the EGF receptor (5 × 10^3 receptors per cell) that facilitates its recovery from the antibody affinity column but do not secrete EGF receptor ligands that could alter the data by unrelated autocrine mechanisms. In addition ECV304 cells are spontaneously immortalized, but they still control the cell cycle and grow in anchorage-dependent condition behaving as a non-transformed cell line.

EGF receptor was recovered by immunoprecipitation of lysates prepared from EGF-treated ECV304 cells and isolated by SDS-PAGE. The peptide mixture obtained from the digested EGF receptor was fractionated on the Fe(III)-IMAC column, and the flow-through from this column was collected in R1, R2, R3, and graphite columns as described in a previous section (Fig. 4). The use of these different columns resulted in a sequence coverage of 71% and detection of 69 different peptides (Table II). Among those, phosphopeptides with m/z 3719.50, 3478.58, 2479.18, 2315.99, 2114.05, 1660.68, and 1290.53 were detected (Figs. 4 and 5, Adh+EGF). MS/MS analysis indicated that the distinct phosphopeptides were phosphorylated on Tyr1045, Tyr1086, Tyr1148, and Tyr1173 as well as on Thr669, Ser967, and Ser1002 (Figs. 3 and 7). These data confirm that the proposed analytical strategy is robust, accurate, and sensitive and allows the detection of EGF-induced sites in the physiological balance between tyrosine kinase and PTPase activation inside the cell.

One of the tyrosine phosphorylated site, Tyr1045, is not a major ligand-induced residue. In fact its phosphorylation was not originally identified on the basis of phosphopeptide mapping but by using site-directed mutagenesis data and inhibitory effects of a synthetic phosphopeptide (33). In contrast, the results reported here provide evidence that the Tyr1045 residue is an EGF-induced phosphorylation site (supplemental data).

**Adhesion-dependent Differential Phosphorylation of EGF Receptor**—A recent report from our group indicates that ty-
Nonspecific kinase receptors such as the EGF receptor require cell-matrix adhesion to activate downstream signaling in response to their ligands (8). To investigate the involvement of integrin-dependent adhesion in EGF-dependent EGF receptor phosphorylation, ECV304 cells were detached from the culture dishes and treated with 50 ng/ml EGF for 30 min in suspension (Fig. 5, Susp + EGF). The EGF receptor purified in this condition was analyzed by MALDI MS and MS/MS, and the results show that, in cells kept in suspension, only the residues Tyr1045, Thr669, Ser967, and Ser1002 were phosphorylated upon EGF treatment (see Fig. 7 for summary). All these assignments were confirmed by MS/MS.

These data indicate that the ability of EGF to induce phosphorylation of specific sites on the EGF receptor in cells detached from the matrix is indeed partial and limited to the specific residue Tyr1045. Therefore, the absence of cell adhesion strongly affects the capacity of the EGF receptor to respond to its physiological ligand. The observation that EGF given to cells in suspension is not able to trigger transcription of early growth genes (8) is consistent with the fact that the EGF receptor is not fully phosphorylated in this condition. Partial phosphorylation of the EGF receptor results in decreased EGF-activated downstream pathways in suspended cells, supporting the concept of anchorage-dependent growth. Indeed treatment of non-transformed cells with growth factors and mitogens in suspension is not able to trigger progression into cell cycle. Whether the lack of cell anchorage to the substratum could modify the structure and the conformation of the EGF receptor, leading to decreased kinase activity, is not known. Lipid composition of membrane domains that contain the EGF receptor and the presence of additional transmembrane proteins that could affect ligand-induced conformation of the EGF receptor should be strictly dependent on cell adhesion. Alternatively increased activation of PTPase activity in suspended cells (34) could also be taken into account as an additional mechanism that can rapidly counteract EGF receptor kinase activation in suspended cells. Moreover our data also show that phosphorylation of EGF receptor on threonine and serine residues is apparently not dependent on cell adhesion because their phosphorylation occurs in adherent as well as in suspended cells. Although the relevance of these phosphorylations is not yet defined, their possible involvement in the presentation and/or maintenance of the receptor on the cell membrane could be hypothesized.

To further investigate the role of cell adhesion in EGF receptor phosphorylation, we decided to analyze ECV304 cells plated on integrin ligands in the absence of EGF. Cells were detached and plated for 30 min on dishes coated with antibodies to the αv integrin, which mimic the interaction with the extracellular matrix, followed by mass spectrometry analysis (Fig. 5, Adh).

Fig. 5 shows the comparison of the MS spectra obtained from the EGF receptor peptides after treatment at three different conditions (Susp + EGF, Adh + EGF, and Adh). Each panel reports the peptides eluted from the various chromatographic columns.

The phosphopeptides corresponding to 663ELVE----ALLR---1081 (m/z 2114.05), 976ALMD----STSR---1007 (m/z 3719.50), and 1045YSSD----SV PK---1075 (m/z 3478.58) were found to be phosphorylated at Thr669, Tyr1045, and Ser1002, respectively, in all three conditions analyzed (Figs. 5, A and B, and 7). In contrast, the phosphopeptides corresponding to 1076RPAG----PA P S R----1107 (m/z 2479.18), 1137GSHQ----FFPK---1155 (m/z 2315.99), and 1157GSTAENAEYLR----1175 (m/z 1290.53) were found to be phosphorylated in cells adherent to integrin ligands with and without EGF stimulation (Fig. 5, B, C, and E). The 1165–1175 peptide was phosphorylated on Tyr1172, and the 1076–1097 peptide was phosphorylated on Tyr1086 under both conditions. Interestingly the MS/MS analysis performed on the 1137–1155 peptide showed that although EGF treatment induces phosphorylation on Tyr1148 (Fig. 6A), cell adhesion triggers phosphorylation on a distinct, different site, namely the Ser1142 (Fig. 6B).

To confirm the differential phosphorylation observed for these two sites, the peptides corresponding to the phosphorylated sites and the corresponding non-phosphorylated form (GSHQISLNDPQDYQDFFPK, GpSHQISLNDPQDYQDFFPK, GSHQIPSLNDPQDYQDFFPK, and GSHQISLNDPpYQDFFPK where pY represents phosphotyrosine and pS is phosphoserine) were synthesized. MALDI-Q-TOF tandem mass spectrometry of each synthetic peptide were recorded and compared with the mass spectrum of the phosphopeptide 1137–1155 eluted from the R1 column, confirming the correct interpretation of the spectrum and annotation of the phosphorylation site. In summary, the adhesion of ECV304 cells for 30 min on dishes coated with antibodies to the αv integrin leads to the detection of phosphorylation on the residues Thr669, Ser967, Ser1002, Tyr1045, Tyr1086, Tyr1086, Tyr1142, and Tyr1173.

Taken together (Fig. 7), all these data indicate that (a) integrin-dependent adhesion is sufficient to trigger ligand-independent phosphorylation of EGF receptor, (b) cell-matrix adhesion is required for the response to EGF, and (c) integrin-dependent adhesion and EGF induce phosphorylation of distinct sites on the EGF receptor. Interestingly Tyr1148, which is a major target of EGF, is not phosphorylated by integrin-dependent adhesion, whereas Ser1142 is not phosphorylated in response to EGF but only by adhesion. Ser1142 is a known substrate of Ca2+/calmodulin-dependent protein kinase II and is involved in EGF receptor internalization and degradation (35). However, a specific functional role for its phosphorylation in integrin-dependent adhesion remains to be defined. Integrin-dependent EGF receptor activation leads to cell survival through the activation of the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase and of the AKT pathway (5, 8) as well as to actin cytoskeleton organization through phosphatidylinositol 3-kinase activation, GTP loading on Vav2, and Rac activation (7). Integrin-dependent adhesion, however, does not trigger cell cycle progression, indicating that the
pathways leading to cell proliferation are not activated. Whether or not the lack of phosphorylation of Tyr\textsuperscript{1148} is relevant to the block of proliferative signaling will be the subject of future investigations. In summary, using a MALDI MS/MS-based analytical approach we were able to reveal the constitutive phosphorylation of Thr\textsuperscript{669}, Ser\textsuperscript{967}, Ser\textsuperscript{1002}, and Tyr\textsuperscript{1045} and the stimulation-induced differential phosphorylation of Tyr\textsuperscript{1068}, Tyr\textsuperscript{1086}, Ser\textsuperscript{1142}, Tyr\textsuperscript{1148}, and Tyr\textsuperscript{1173} (Fig. 7).
Stimulation-dependent Multisite Phosphorylation of the EGF Receptor

Mass spectrometry was used previously for studies of phosphorylation of the EGF receptor. Salek et al. (36) used immortalized keratinocytes (HaCaT) that express 10^6 EGF receptors per cell to selectively detect the tyrosine phosphorylation at Tyr^{1086}, Tyr^{1148}, and Tyr^{1173} in the EGF receptor. In another study, phosphotyrosine-specific immumion ion scanning by nanoelectrospray MS/MS was applied to characterize several phosphoproteins in the EGF receptor signal transduction pathway in HeLa cells, including the detection of phosphorylation at Tyr^{1148} and Tyr^{1173} (37). Anti-phosphotyrosine and anti-EGF receptor affinity chromatography, isotope-coded micro-LC-MS/MS mass spectrometry and immunoblot methods were applied. Guo et al. (38) used LC-MS/MS to study a human epidermoid carcinoma cell line (A431) in which the EGF receptor was overexpressed. The cells were untreated or treated with EGF or transforming growth factor α at different concentrations. Seven different phosphorylation sites were detected (Tyr^{992}, Tyr^{1045}, Tyr^{1068}, Ser^{1142}, Tyr^{1148}, and Tyr^{1173}), and one doubly phosphorylated peptide containing Tyr^{1148} and Ser^{1142} was found. The pattern of EGF receptor phosphorylation (Thr^{669}, Ser^{967}, Tyr^{1045}, Tyr^{1068}, Tyr^{1086}, Ser^{1142}, Tyr^{1148}, and Tyr^{1173}) detected by us is similar to what was determined by Guo et al. (38). In addition we were also able to find and confirm phosphorylation on a novel site, namely Ser^{967}. Recent studies of phosphotyrosine signaling networks in squamous carcinoma cells that overexpressed EGF receptor (5 × 10^6 EGF receptors per cell) identified phosphopeptides that contain Ser(P)^{967}, Tyr(P)^{974}, Tyr(P)^{1086}, Tyr(P)^{1114}, Tyr(P)^{1148}, and Tyr(P)^{1173} (39).

Conclusion—We have demonstrated a MALDI MS- and MS/MS-based analytical strategy targeted toward the detection and sequencing of phosphopeptides derived from the EGF receptor that was purified using appropriate affinity chromatography. We were able to map a majority of the known phosphorylation sites and identify a novel site on the EGF receptor. This approach should be generally applicable for analysis of phosphoproteins that are selectively immunoprecipitated, it is compatible with SDS-PAGE separation, and it utilizes a simple set-up for sample preparation.

Affinity-based purification techniques combined with fractionation of complex peptide mixtures, enzyme treatment, and MS/MS analysis were useful for phosphopeptide mapping and sequencing of a large protein such as the EGF receptor. The method used for peptide mixture sample preparation prior to mass spectrometry analysis was a critical factor to improve the detection of phosphopeptides. The use of three distinct reverse phase chromatography materials (POROS R1, R2, and R3 resins), graphite powder, and the selective enrichment of phosphopeptides using IMAC allows the handling of subpicomole amounts of peptides and minimizes the sample losses by recovering a large portion of the tryptic peptides and phosphopeptides for MALDI MS and MS/MS analysis. The high mass resolution and mass accuracy of the MALDI-Q-TOF instrument allowed the exact localization of phosphorylated amino acid residues in phosphopeptides up to 3400 Da.

In the present implementation of our protocol, mass spectrometry was used to obtain qualitative and semiquantitative information about the major phosphorylated residues in the EGF receptor subjected to various cellular stimuli. This approach can be further refined by using stable isotope labeling prior to mass spectrometry analysis to achieve more accurate quantitation of dynamic phosphorylation and dephosphorylation events (30, 40). The combination of miniaturized sample preparation technology and high throughput MALDI MS/MS with large scale immunoprecipitation of proteins (41) potentially provides a platform for systematic analysis of cell signaling pathways in resting and perturbed states.

Acknowledgments—We thank Dr. Waltraud Schulze for assisting during preparation of synthetic phosphopeptides and Dr. Jakob Bunkenborg and Dr. Martin Larsen for assistance in mass spectrometry.

* This work was supported by grants from the Italian Association for Cancer Research (AIRC) (to P. D.), MIUR (Ministero dell’Università e
Ricerca Scientifica, cofinanziamento Italian Ministry of University, Technological and Scientific Research and fondi ex-60% and Italian basic research funds (Fondo di Incentivazione ne della Ricerca di Base) (FIRB) (to P. D. and G. T.), and special project “OncoLogic,” Compagnia San Paolo/International Foundation for Experimental Medical Research (Fondazione internazionale di ricerca in medicina sperimentale) (FIRMS), Torino, Italy (to P. D. and G. T.). Research in the Protein Research Group is supported by grants from the Danish Research Agency (to Danish Biotechnology Instrument Center) and by the Danish Natural Sciences and Technical Sciences Research Councils. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

† Supported by a Marie Curie training fellowship from the European Union commission and an Agency for the Internationalisation of Education and Training (Center for information og rådgivning om international e uddannelses –og Samarbejds aktiviteter) (CIRIUS) fellowship from the Danish government.

§ To whom correspondence may be addressed. Tel.: 39-011-6705885; Fax: 39-011-6705853; E-mail: paola.defilippi@unito.it.

** A Lundbeck Foundation research professor. To whom correspondence may be addressed. Tel.: 39-011-9405–9414.

REFERENCES


25. Deleted in proof


31. Boeri Erba, E., Bergatto, E., Cabodi, S., Silengo, L., Tarone, G., Defilippi, P.,


