Analysis of receptor signaling pathways by mass spectrometry: Identification of Vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors

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Contributed by Harvey F. Lodish, October 29, 1999

Oligomerization of receptor protein tyrosine kinases such as the epidermal growth factor receptor (EGFR) by their cognate ligands leads to activation of the receptor. Transphosphorylation of the receptor subunits is followed by the recruitment of signaling molecules containing src homology 2 (SH2) or phosphotyrosine interaction domains (PID). Additionally, several cytoplasmic proteins that may or may not associate with the receptor undergo tyrosine phosphorylation. To identify several components of the EGFR signaling pathway in a single step, we have immunoprecipitated molecules that are tyrosine phosphorylated in response to EGF and analyzed them by one-dimensional gel electrophoresis followed by mass spectrometry. Combining matrix-assisted laser desorption/ionization (MALDI) and nanoelectrospray tandem mass spectrometry (MS/MS) led to the identification of nine signaling molecules, seven of which had previously been implicated in EGFR signaling. Several of these molecules were identified from low femtomole levels of protein loaded onto the gel. We identified Vav-2, a recently discovered guanosine nucleotide exchange factor that is expressed ubiquitously, as a substrate of the EGFR. We demonstrate that Vav-2 is phosphorylated on tyrosine residues in response to EGF and associates with the EGFR *in vivo*. Binding of Vav-2 to the EGFR is mediated by the SH2 domain of Vav-2. In keeping with its ubiquitous expression, Vav-2 seems to be a general signaling molecule, since it also associates with the platelet-derived growth factor (PDGF) receptor and undergoes tyrosine phosphorylation in fibroblasts upon PDGF stimulation. The strategy suggested here can be used for routine identification of downstream components of cell surface receptors in mammalian cells.

Cells respond to a variety of extracellular stimuli such as growth factors, osmotic stress, hormones, and nutritional deprivation. Many of these responses are mediated by cell surface receptors, and activation of these receptors can trigger cellular proliferation, differentiation, or cytoskeletal changes (1). Alteration in cellular phosphorylation is a major mechanism for the flow of information from the outside of a cell to the inside. Receptor protein tyrosine kinases are an important class of cell surface receptors that mediate such signals (2). In this study, we sought to discover additional components of a prototypical receptor tyrosine kinase—the epidermal growth factor receptor (EGFR). Oligomerization of the EGFR is followed by an increase in catalytic activity of the receptor. This activity results in tyrosine phosphorylation of the receptor itself as well as of several cytoplasmic signaling molecules. Numerous such substrates have already been identified, and many of these contain phosphotyrosine-binding domains such as src homology 2 (SH2) and phosphotyrosine interaction domains (PID) (3). Tyrosine phosphorylation of some of these substrates such as phospholipase C γ (PLC-γ) leads to increased enzymatic activity contributing to receptor-mediated signaling cascade (4, 5).

Edman sequencing has traditionally been used for identification after biochemical purification of molecules. However, this approach requires large amounts of material and precludes discovery of substrates that may be present at low levels in the cell. With the recent advances in mass spectrometry and the explosive growth of sequence databases, it has become possible to use femtomole amounts of proteins from gels for identification (6, 7). We have previously suggested the use of mass spectrometry for the systematic study of multiprotein complexes obtained after affinity-based purification, and the definition of the yeast U1 small nuclear ribonucleoprotein (snRNP) complex demonstrated the utility of these mass spectrometric methods (8, 9). Examples of such studies include isolation of spliceosome complex, anaphase-promoting complex, and profilin-associated proteins (10–12). Here we show that straightforward mass spectrometric analysis can define a number of proteins in a signal transduction pathway—receptor-associated proteins as well as other downstream molecules. To identify components of the EGFR signaling complex, we used lysates from unstimulated and EGF-stimulated HeLa cells for immunoprecipitation with anti-phosphotyrosine antibodies. The immunoprecipitates were resolved by one-dimensional electrophoresis and the proteins of interest were excised from silver-stained gels. After in-gel digestion by trypsin, the samples were analyzed by matrix-assisted laser desorption/ionization (MALDI) and nanoelectrospray tandem mass spectrometry (MS/MS).

A study describing analysis of proteins that are phosphorylated in response to platelet-derived growth factor (PDGF) in NIH 3T3 fibroblasts by using anti-phosphotyrosine and anti-phosphoserine antibodies was published recently (13). Whole-cell lysates from unstimulated and PDGF-stimulated cells were resolved by two-dimensional electrophoresis followed by immunoblotting to detect proteins. Although 41 proteins were inducibly phosphorylated on tyrosines (as detected by immunoblotting with anti-phosphotyrosine antibodies), only a fraction of them were identified by mass spectrometry. In their report, Soskic *et al.* (13) failed to identify any novel proteins or any of the several

Abbreviations: EGFR, epidermal growth factor receptor; GST, glutathione *S*-transferase; MALDI, matrix-assisted laser desorption/ionization; MS/MS, tandem mass spectrometry; PDGF, platelet-derived growth factor; PID, phosphotyrosine interaction domain; SH2, src homology 2; SH3, src homology 3.

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well described substrates of the PDGF receptor that contain an SH2 or PID. Because signaling molecules are usually present at low abundance in cells, we first enriched for tyrosine-phosphorylated molecules by an immunoprecipitation step followed by one-dimensional electrophoresis. We opted to use one-dimensional electrophoresis instead of two-dimensional gels because we were unable to routinely detect any proteins more than about 100 kDa on two-dimensional gels. A strategy similar to ours has been described earlier (14) in which anti-phosphotyrosine immunoprecipitates from a B cell lymphoma line activated by anti-IgM were subjected to ion-exchange chromatography, followed by SDS/PAGE and transfer to an Immobilon membrane. Proteins were digested on the membrane and the peptides were separated by reverse-phase HPLC before being analyzed by mass spectrometry. Only one known signaling molecule, Syk, was identified in this study. The major disadvantage of such an approach, besides that it involves a number of preparative chromatographic steps, is that proteins that are tyrosine phosphorylated in the basal state as well as those that bind nonspecifically to the column will also be enriched.

Our strategy resulted in the isolation of two additional candidates as well as several known second messengers in the EGFR signaling pathway. Most of these were identified both by MALDI and MS/MS, whereas others could not be identified with certainty by MALDI alone. Protein mixtures migrating as a single band on the gel were also easily characterized. Several proteins were present in femtomole amounts–e.g., the p85 subunit of phosphatidylinositol 3-kinase was identified from less than 100 fmol of protein present in the gel. A recently discovered guanosine nucleotide exchange factor, Vav-2 (15, 16), was found to be a substrate of the EGFR. Because Vav-2 is ubiquitously expressed, it had been hypothesized that it may be downstream of receptor tyrosine kinases in nonhematopoietic cells (16). In this report we show that Vav-2 is indeed tyrosine phosphorylated in response to EGF. We demonstrate that Vav-2 associates with the EGFR *in vivo* and that the SH2 domain of Vav-2 mediates this interaction. We find that Vav-2 is expressed in fibroblasts and is also a substrate of the PDGF receptor, another member of the receptor tyrosine kinase superfamily. Finally, we have cloned another protein that is tyrosine phosphorylated upon EGF treatment whose role in the EGFR signaling pathway remains to be investigated.

Materials and Methods

Cell Culture and Antibodies. HeLa S3 cells were grown in Joklik's MEM with 5% fetal bovine serum and 2 g/liter $NaHCO_3$. The cells were grown to a density of 10^6 per ml and then cultured for an additional 15 hr without serum. NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum. A mixture of anti-phosphotyrosine antibodies was used in immunoprecipitation experiments: 4G10 mouse monoclonal antibody from Upstate Biotechnology (Lake Placid, NY) and RC20 monoclonal antibody from Transduction Laboratories (Lexington, KY). Anti-EGFR monoclonal antibody was purchased from Upstate Biotechnology. The Vav-2 rabbit polyclonal antibody was raised against a synthetic peptide corresponding to amino acid residues 208–222 of human Vav-2 and does not crossreact with Vav. EGF and PDGF were purchased from Upstate Biotechnology.

Immunoprecipitation and Western Blotting. For experiments described in Fig. 1, a total of 5×10^9 serum-deprived HeLa S3 cells were left untreated or treated with 1 μg/ml of EGF for 5 min. The cells were lysed in 25 ml of lysis buffer containing 50 mM Tris·HCl at pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and 1 mM sodium orthovanadate in the presence of protease inhibitors. Cleared cell lysates were incubated with 100 μg of 4G10 monoclonal antibody coupled to agarose beads and 50 μg of biotin-

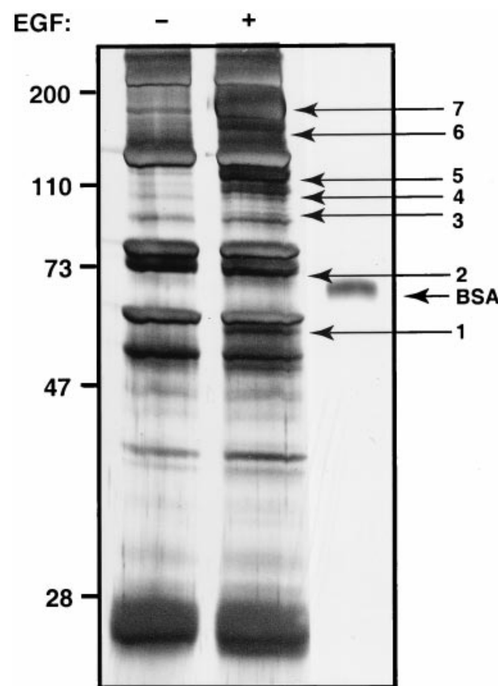

Fig. 1. EGF-induced tyrosine phosphorylation in HeLa cells. Serum-deprived HeLa S3 cells (5×10^9) were either left untreated or treated with 1 μg/ml EGF for 5 min. Cleared cell lysates were immunoprecipitated with a mixture of monoclonal anti-phosphotyrosine antibodies, washed, and resolved by SDS/PAGE. The gel was then silver-stained. Molecular mass markers in kDa are indicated as well as 150 fmol of BSA that was loaded onto the same gel. The arrows with numbers indicate the positions of the bands that were excised for enzymatic digestion by trypsin and subsequent mass spectrometric analysis.

conjugated RC20 antibody bound to streptavidin-agarose beads for 4 hr at 4°C. Precipitated immune complexes were then washed three times with lysis buffer, boiled in sample buffer, and resolved by SDS/PAGE. The gels were silver-stained as previously described (17).

For coimmunoprecipitation studies, 4×10^6 HeLa cells were grown and starved as above. They were treated with 100 ng/ml EGF for 5 min and lysed. Also, 5×10^6 NIH 3T3 fibroblasts were grown and starved as above and treated with 50 ng/ml PDGF. Cleared cell lysates were immunoprecipitated with 5 μl of anti-Vav-2 polyclonal antiserum for 2 hr at 4°C. After incubation, the beads were washed three times in lysis buffer, boiled in sample buffer, resolved by SDS/PAGE, and transferred onto nitrocellulose. The membrane was blocked with 1% BSA in phosphate-buffered saline containing 0.1% Tween-20 overnight at 4°C and then incubated with 1 μg/ml 4G10 anti-phosphotyrosine antibody for 2 hr. The membranes were incubated with secondary antibodies followed by reagents for chemiluminescent detection according to the manufacturer's instructions (ECL, Amersham).

Glutatione *S*-Transferase (GST)-Binding Assays. A 550-bp fragment encoding the SH2 domain of Vav-2 (amino acids 655–819) was subcloned into pGEX4T-3 (Pharmacia). A 564-bp fragment encoding the C-terminal SH3 domain of Vav-2 (amino acids 719–868) was subcloned into pGEX5X-2. For GST-binding assays, HeLa cells were grown and lysates from 4×10^6 unstimulated and EGF-stimulated cells were prepared as mentioned above. Cleared cell lysates were incubated with 10 μg of GST alone, GST-SH2 or GST-SH3 (C-terminal) fusion proteins bound to glutathione-agarose beads for 2 hr at 4°C. After incubation, beads were washed three times in lysis buffer, boiled in SDS sample buffer, resolved by

Table 1. Summary of identification of proteins from the gel in Fig. 1

Protein band analyzed	Molecule identified	Identification by MALDI	Number of peptide matches	Sequence coverage, %	Identification by MS/MS
1	Shc	+	25	40	ND
2	p62	−	0	0	+
3	p85	+	26	32	+
4	Vav-2	+	18	26	+
	Hrs	−	12	14	+
5	Hrs	+	22	19	+
	Cbl	+	30	38	+
	Eps15	−	14	14	+
6	Novel protein	+	37	35	+
	EGFR	+	22	21	+
7	EGFR	+	43	36	ND

Gel slices from the silver-stained gel shown in Fig. 1 labeled 1 through 7 were excised, digested with trypsin, and subjected to MALDI mass spectrometry followed by nanoelectrospray MS/MS. This table lists the molecules identified from each gel slice. Some proteins were identified from more than one gel slice. Number of tryptic peptides observed by MALDI analysis that matched tryptic peptides derived from a given protein in the database is shown. Sequence coverage refers to the percent of a given protein that corresponded to the tryptic peptides detected by MALDI analysis. ND, not done.

SDS/PAGE, and transferred onto nitrocellulose. The membrane was subjected to Western blotting as described above, using an anti-phosphotyrosine antibody.

Mass Spectrometry. Mass spectrometric identification of proteins was done according to the strategy previously described (6). Briefly, the protein bands were excised from one-dimensional silver-stained polyacrylamide gel shown in Fig. 1 and processed as described (6). After reduction and alkylation, proteins were digested in the gel with an excess of sequencing-grade trypsin (Boehringer Mannheim). The digestion was carried out for 4 hr at 37°C. Samples for MALDI analysis were prepared as described (18) by using the "fast evaporation" method (19). Mass spectra were recorded on a Bruker Reflex III MALDI time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). Matrix-related ions and trypsin autolysis products were used for internal calibration. Delayed ion extraction resulted in peptide masses with better than 30 ppm mass accuracy on average, limited mainly by ion statistics in the smaller peaks. PEPSEA software (Protana, Odense, Denmark) was used to search a nonredundant protein sequence database (nrdb; searchable at the National Center for Biotechnology Information) with a list of peptide masses. Protein mixtures were resolved by MALDI analysis using an iterative algorithm described earlier (20).

The samples for MS/MS analysis were prepared essentially as described (17). After in-gel digestion, 0.3 μl of supernatant (1–3% of the total of about 30 μl) was removed for MALDI analysis and the rest was loaded onto a Poros R2™ (PerSeptive Biosystems, Framingham, MA) microcartridge (21), desalted, and eluted into nanoelectrospray needles (Protana). Nanoelectrospray MS/MS analysis (22) was performed on a prototype of a quadrupole time-of-flight mass spectrometer (23) (QSTAR; Sciex, Toronto, Canada), and fragmentation spectra were obtained for as many peptides as possible. The resulting "peptide sequence tags" (24) were used to search the nrdb database. After a peptide match was found in the database, the retrieved peptide sequence was verified against the MS/MS spectrum.

Results and Discussion

Identification of Multiple EGFR Substrates by SDS/PAGE and Mass Spectrometry. To identify substrates of the EGFR, we used a mixture of anti-phosphotyrosine antibodies to immunoprecipitate proteins that are phosphorylated on tyrosines in response to EGF. HeLa S3, a human cervical carcinoma cell line, was chosen for this purpose because it expresses high levels of the EGFR and can be grown in suspension. Moreover, because the expressed sequence tag database (dbEST) is almost three times larger for humans than for mouse or any other species (25), it is possible to use peptide sequence tags to increase the likelihood of finding ESTs if the data are derived from a human protein. Fig. 1 shows a silver-stained gel of immunoprecipitates from a mixture of anti-phosphotyrosine antibodies. The bands that are seen exclusively in the EGF-treated lane may either be directly tyrosine phosphorylated by the EGFR or undergo tyrosine phosphorylation induced by a downstream tyrosine kinase. In addition, some of these proteins may not be tyrosine phosphorylated but may bind to another protein that is tyrosine phosphorylated by EGFR. We chose to resolve proteins by one-dimensional electrophoresis because we could not reproducibly observe proteins greater than 100 kDa in analytical two-dimensional electrophoretic gels. Polyacrylamide gel slices from SDS/PAGE gels corresponding to tyrosine-phosphorylated proteins were excised and subjected to in-gel digestion by trypsin. After digestion, a small portion of the supernatant was removed and analyzed by high-accuracy peptide mass mapping using MALDI. The peptide masses obtained by MALDI analysis were used to search protein databases. If the identification of the protein was ambiguous after MALDI analysis, or if there was a mixture of proteins in the sample, the remainder of the sample was analyzed by nanoelectrospray MS/MS. This provided a partial amino acid sequence of the peptide, thereby confirming the assignment by MALDI or indicating that the protein in question was novel. We routinely observe peaks that correspond to keratin, presumably resulting from contamination during the handling of gel slices. Such peaks were excluded from our analyses.

Analysis of Complex Mixtures by Mass Spectrometry. Table 1 lists the proteins identified from the gel shown in Fig. 1. Seven previously known substrates and two novel substrates were identified in this strategy. Four of the bands excised from the gel contained a single identifiable component of the EGFR signaling pathway each. These proteins were Shc (band 1), p62 (band 2), p85 subunit of phosphatidylinositol 3-kinase (band 3), and EGFR itself (band 7). All of these are bona fide substrates of the EGFR and are known to undergo tyrosine phosphorylation upon activation of the EGFR. p85 subunit contains two SH2 domains, whereas Shc contains both SH2 and phosphotyrosine interaction domains (26–29); both of these proteins associate with the activated EGFR. Although p62 does not contain an SH2 domain, it contains several SH3-binding motifs. Upon tyrosine phosphorylation, it associates with ras GTPase-activating protein (ras-

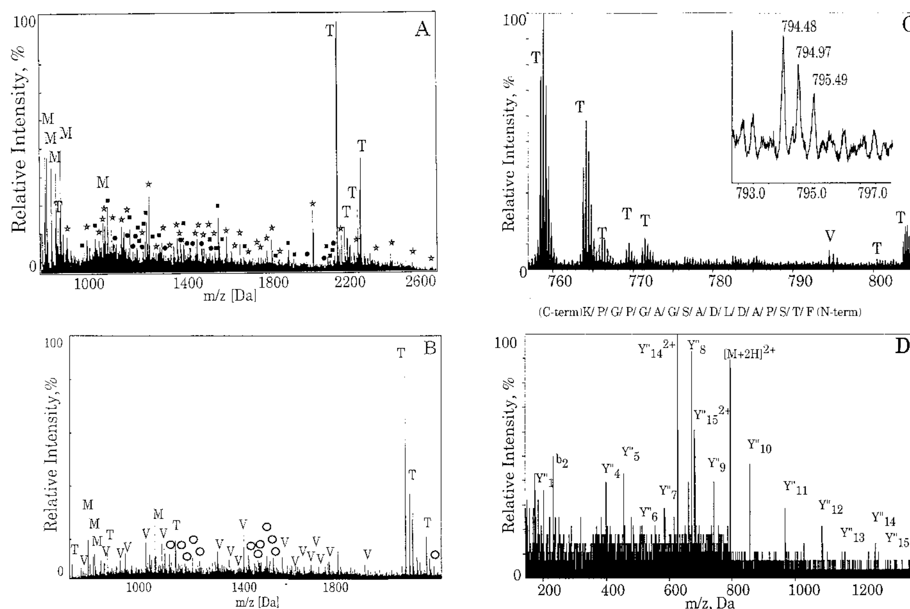

Fig. 2. Use of mass spectrometry to identify Vav-2 and to resolve protein mixtures. Proteins separated by SDS/PAGE shown in Fig. 1 were subjected to in-gel digestion by trypsin and analyzed by mass spectrometry. (*A*) Protein band 6 was analyzed by MALDI. The tryptic peptides from the digest indicate the presence of several proteins in this mixture. Filled squares correspond to peptides derived from EGFR, T refers to trypsin autolysis products, and M denotes matrix ions. The peaks marked with stars denote a novel protein. Peaks marked with filled circles correspond to Eps15 (confirmed by MS/MS). (*B*) Protein band 4 was analyzed by MALDI. The tryptic peptides from this band showed the presence of peptides corresponding to Vav-2 (labeled V) and Hrs (shown by open circles). T refers to trypsin autolysis products and M denotes matrix ions. (*C*) MS spectrum from nanoelectrospray MS/MS analysis of the peptides from the sample analyzed by MALDI in *B*. V shows a peak corresponding to Vav-2, and T refers to trypsin autolysis products and their sodium adducts. (*Inset*) Isotopic resolution of a doubly charged peptide corresponding to Vav-2. (*D*) Fragmentation of the doubly charged peptide ($[M+2H]^2$) shown in *C* (m/z = 794.48) by MS/MS. The Y″ series of ions (C-terminal fragments) that are produced due to fragmentation are shown as well as one from the B series (N-terminal fragments; b_2). The sequence of the peptide derived from this spectrum is shown at the top of the panel.

GAP), phospholipase C γ (PLC-γ), and Grb2 (30–32). The remaining three bands excised from the gel contained more than one protein each. This was evident both by MALDI analysis of tryptic peptides derived from these mixtures and by nanoelectrospray MS/MS experiments. For instance, Fig. 2*A* shows the MALDI spectrum of band 6. The peaks correspond to tryptic peptides derived from EGFR and other proteins (marked by stars). Using an iterative strategy for resolving peptide mixtures from different proteins (20), EGFR and a novel protein, which was deposited in the public database as an mRNA fragment, were identified first. A third round of searching assigned 14 peaks to Eps15 with 14% sequence coverage. However, the MALDI data alone were not sufficient to unambiguously identify this protein as Eps15. Sequencing of peptides by nanoelectrospray MS/MS analysis confirmed the presence of Eps15. This protein is an Eps15 homology (EH) domain-containing protein that was previously found in a similar strategy to isolate molecules that were tyrosine phosphorylated in response to EGF (33). Analysis of band 5 showed that it also contained a mixture of proteins—Hrs and Cbl. Hrs is a 115-kDa protein with a zinc finger domain and a proline-rich region (34). It undergoes phosphorylation on tyrosines in response to EGF and a variety of other growth factors. Cbl is an adapter protein that exists in a complex with Grb2 in its basal state and upon EGF treatment associates with the EGFR as well as other cytoplasmic signaling molecules, including Shc, p85, and CrkL (35, 36).

The analysis of band number 4 was complicated by the close migration to band number 5, and the MALDI peptide spectrum indicated the presence of two proteins. Besides the main component Vav-2, a protein present in band 5, Hrs, was also identified (Fig. 2*B*). To characterize this protein mixture in more detail and search for possible additional components, the remainder of the peptide material (98%) was transferred to a nanoelectrospray needle. Fig. 2*C* shows an MS spectrum obtained during nanoelectrospray mass spectrometric analysis of band 4. The isotopic resolution shown in the *Inset* allows one to easily distinguish multiply charged peptides from singly charged chemical noise even if the signal is weak. In this instance, the peptide corresponding to m/z ratio of 794.48 was chosen for further fragmentation and sequencing. Fig. 2*D* shows the MS/MS spectrum that matched a peptide derived from Vav-2. Sequencing of several peptides from the mixture confirmed the presence of Vav-2 and HRS but did not reveal any other protein component.

EGF Treatment Leads to Tyrosine Phosphorylation of Vav-2. Vav-2 is a recently discovered protein that exhibits 55% identity at the protein level to a previously described molecule designated Vav (15, 16). Vav contains a Dbl homology domain that is found in the Rho/Rac family of GTP-binding proteins, one SH2 domain, two SH3 domains, and an N-terminal leucine-rich region (37). The expression of Vav is restricted to cells of the hematopoietic lineage. Phosphorylation of Vav occurs on tyrosine residues after activation of several receptors, including B and T cell receptors, c-Kit receptor, erythropoietin receptor, and granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor (38–43). This phosphorylation increases the GDP/GTP exchange activity toward Rac-1 (44). Gene targeting experiments have shown Vav to be critical during the implantation of mouse embryos as well as for proliferative responses in T and B cells (45–48).

In contrast to the limited expression of Vav, Vav-2 is expressed ubiquitously (16). It has been proposed to act in a manner similar to that of Vav. First, an N-terminal-deleted version of Vav-2 is able to transform rodent fibroblasts although the cells transformed by Vav-2 have a different morphology than those

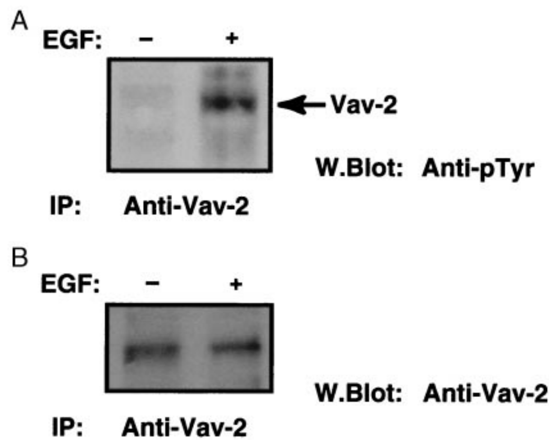

Fig. 3. Vav-2 is tyrosine phosphorylated by EGF treatment. HeLa cells were either left untreated or treated with EGF for 5 min, and lysates were immunoprecipitated (IP) with anti-Vav-2 antibody as indicated. Washed immunoprecipitates were resolved by SDS/PAGE, transferred onto nitrocellulose, and Western blotted with an anti-phosphotyrosine antibody (*A*). The position of tyrosine-phosphorylated Vav-2 is indicated by an arrow. *B* shows equal loading of Vav-2 from a parallel experiment.

transformed by oncogenic Vav (16). Second, coexpression of Vav-2 and an activated tyrosine kinase, lck, leads to activation of wild-type Vav-2 (49). This observation suggests that phosphorylation of Vav-2 by endogenous tyrosine kinases such as receptor tyrosine kinases can lead to its activation as well.

As shown in Fig. 3, Vav-2 undergoes tyrosine phosphorylation in response to EGF stimulation. This demonstration that Vav-2 can be phosphorylated in response to a physiologic stimulus explains why we were able to immunoprecipitate Vav-2 by anti-phosphotyrosine antibodies from EGF-stimulated HeLa cells but not from untreated cells (Fig. 1).

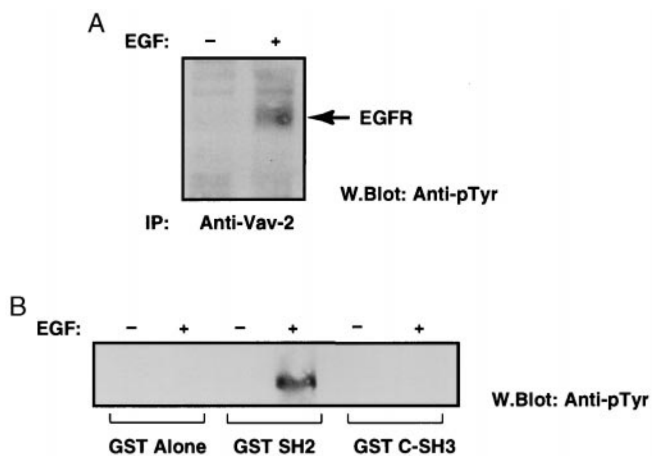

Fig. 4. Vav-2 associates with EGFR by means of its SH2 domain. (*A*) HeLa cells either were left untreated or were treated with EGF, and lysates were immunoprecipitated with anti-Vav-2 antibody as indicated. Washed immunoprecipitates were resolved by SDS/PAGE, transferred onto nitrocellulose, and Western blotted with an anti-phosphotyrosine antibody. Coimmunoprecipitated EGFR is indicated by an arrow. (*B*) HeLa cells either were left untreated or were treated with EGF, and lysates were incubated with 10 μg of GST alone, GST-SH2, or GST-SH3 (C-terminal) fusion proteins bound to glutathione-agarose beads for 2 hr at 4°C. The beads were then washed and boiled in sample buffer. The samples were resolved by SDS/PAGE, transferred onto nitrocellulose, and Western blotted with an anti-phosphotyrosine antibody to detect the EGFR.

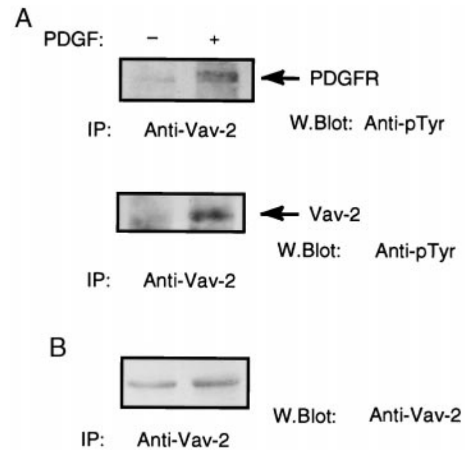

Fig. 5. Vav-2 is a substrate of the PDGF receptor in fibroblasts. (*A*) NIH 3T3 fibroblasts either were left untreated or were treated with PDGF, and lysates were immunoprecipitated with anti-Vav-2 antibody as indicated. Washed immunoprecipitates were resolved by SDS/PAGE, transferred onto nitrocellulose, and Western blotted with an anti-phosphotyrosine antibody. Coimmunoprecipitated PDGFR and Vav-2 are indicated by arrows. (*B*) Anti-Vav-2 immunoprecipitates from a parallel experiment to show equal loading of Vav-2.

Vav-2 Associates with the EGFR by Means of Its SH2 Domain. To address whether Vav-2 associates with the EGFR, we immunoprecipitated endogenous Vav-2 from unstimulated and EGF-stimulated HeLa cells and then used Western blotting to detect EGFR. Fig. 4*A* shows that Vav-2 associates with the EGFR only upon EGF treatment. Because this behavior is reminiscent of a phosphotyrosine-based interaction, we wished to determine whether the SH2 domain of Vav-2 mediates this interaction. As shown in Fig. 4*B*, the SH2 domain, but not the C-terminal SH3 domain or GST alone, precipitated EGFR from EGF-stimulated HeLa cells. Thus, we have demonstrated that Vav-2 associates with the EGFR by means of its SH2 domain and that it undergoes phosphorylation on tyrosines in response to EGF treatment

Vav-2 Is Expressed in Fibroblasts and Is Also a Substrate for the PDGF Receptor. To examine whether Vav-2 is a general signaling molecule that is involved in other pathways, we chose NIH 3T3 cells because they are fibroblasts, in contrast to HeLa cells, which are epithelial in origin. These cells exhibit a mitogenic response to PDGF that is a ligand for the PDGF receptor, a tyrosine kinase containing an insert in its kinase domain. We found that Vav-2 is expressed in these cells and undergoes tyrosine phosphorylation and associates with the endogenous PDGF receptor in a ligand-dependent manner (Fig. 5). This finding shows that Vav-2 is not specific for the EGFR signaling pathway but is a general signaling intermediate that is involved in other signal transduction pathways as well.

Conclusions. We have described the use of a single-step affinity-based purification followed by one-dimensional electrophoresis and mass spectrometry to identify several molecules involved in EGFR signaling. Our objective was to isolate components of the EGFR signaling pathway, and tyrosine phosphorylation of such substrates was used as the basis of the purification scheme. This approach is unbiased because no other assumption was made about the downstream molecules. This strategy has advantages over other methods to isolate interacting molecules such as the yeast two-hybrid system for several reasons: First, it identifies molecules on the basis of modifications *in vivo* and therefore requires less experimentation to validate the finding once a molecule is identified. Second, this strategy does not require direct interaction of the receptor with the

downstream molecule. For instance, in this report we have isolated molecules that directly interact with the EGFR as well as others that do not associate with the receptor. Third, this strategy has the potential of identifying multiple signaling components in one step. In view of the simplicity of this experiment, it is surprising that we were able to identify many key components of the EGF receptor pathway that were originally discovered over the course of a decade by several groups using a variety of experimental approaches. This strategy can be easily extended to several other receptor-mediated signaling pathways, including other receptor tyrosine kinases, cytokine receptors, and B and T cell receptors whose activation results in tyrosine phosphorylation of cellular substrates.

We thank members of the Protein Interaction Laboratory for helpful discussions and Scott Waniger and Mark Hirschel at the National Cell Culture Center, Minneapolis (sponsored by the National Center for Research Resources, Comparative Medicine, National Institutes of Health) for growing HeLa S3 cells. We thank Drs. Peter Roepstorff, Karsten Kristiansen, Olaf-Georg Issinger, and Ole Jensen for a critical reading of this manuscript. We are also grateful to Doris Petersen and Nina Krogh for their help with figures. A.P. was supported by the Howard Temin Award from the National Cancer Institute (KO1 CA75447). H.F.L. is supported by PO1 HL32262 from the National Heart, Lung, and Blood Institute. Work at the Protein Interaction Laboratory was supported by a grant from the Danish National Research Foundation to the Center for Experimental Bioinformatics.

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