Subsets of Epidermal Growth Factor Receptors during Activation and Endocytosis

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David R. Emlet‡, David K. Moscatellο§, Laural B. Ludlow§, and Albert J. Wong‡§¶
From the Department of ¶Pharmacology and the §Department of Microbiology and Immunology, Kimmel Cancer Institute, Philadelphia, Pennsylvania 19107

Mutation of the autophosphorylation sites of receptor protein-tyrosine kinases alters ligand dependent internalization and down-regulation, indicating a critical role for these sites in receptor processing. Currently, no differences in receptor processing based on an individual autophosphorylation site have been defined. By using a glutathione S-transferase fusion protein containing the src homology 2 domains of phospholipase C-γ2 to specifically recognize tyrosine 992 on the EGF receptor (Tyr(P)992), we have found differences in this subpopulation of receptors. Following EGF stimulation, the number of Tyr(P)992 receptors increased 2-fold over receptors identified by an antibody that recognizes activated EGF receptor; PLC-γ1, phospholipase C-γ1 containing the SH2 domains of PLC-γ1, and SHPTP2, a tyrosine phosphatase, was associated with more SOS, Ras-GTPase-activating protein, but less Grb2, than receptor processing, another inference is that intact receptors may undergo differential processing based on which sites are phosphorylated, although this has never been directly visualized on intact receptors in situ.

Several studies have now shown that receptor internalization plays a role in signaling. Upon activation, receptors are endocytosed from the cell surface, transferred to endosomes, and then multivesicular bodies, which ultimately fuse with lysosomes (5, 14, 15). Because the autophosphorylation sites and kinase domain of the receptor are oriented toward the cytoplasm during endocytosis (2, 16), the receptor can continue to signal until it is degraded in the lysosomal compartment (16–18). The EGF receptor kinase domain remains highly active during endocytosis (16), and certain substrates are phosphorylated following internalization. Futter et al. (19) have shown that annexin I is preferentially phosphorylated in the multivesicular body by the EGF receptor. A 55-kDa protein now identified as She has been shown to associate with the receptor in both membrane and endosomal fractions (20).

One implication of these findings is that receptor autophosphorylation sites could have a role in receptor internalization and degradation. Since the five autophosphorylation sites on the EGF receptor are not phosphorylated to the same extent (3, 21), there would be differential interaction of the EGF receptor with the SH2 domain containing effectors. Furthermore, since mutation of an individual autophosphorylation site can affect processing, another inference is that intact receptors may undergo differential processing based on which sites are phosphorylated, although this has never been directly visualized on intact receptors in situ.

We have found that an SH2 domain fusion protein can identify an EGF receptor population that exhibits different rates of endocytosis, kinetics of activation, effector protein interaction, and level of phosphorylation than the general pool of EGF receptors. Phospholipase C-γ1 contains two adjacent SH2 domains that bind to Tyr(P)992 in the EGF receptor and maintain their specificity for this single-site even when used at higher molar concentrations (22). In this study, we produced a GST fusion protein of the SH2 domains of PLC-γ1 (PLC-SH2), and used this to identify Tyr(P)992 EGF receptors during the process of ligand internalization and internalization in A431 cells, a human epidermoid carcinoma cell line that overexpresses the EGF receptor.

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Experimental Procedures—The two SH2 domains of PLC-γ1 (nucleotides 1697–2468) (23) were subcloned into pELEX-5x-3 (Pharmacia Biotech Inc.). The GST fusion protein (PLC-SH2), was produced and purified as described by the manufacturer. Protein was dialyzed overnight against PBS, 5 mM dithiothreitol (24) at 4 °C and concentrated in a Centri-60 min with 0.2% Triton X-100 in PBS; blocked in PBS, 5% nonfat dry milk. A monoclonal antibody against phosphotyrosine (Transduction Laboratories) was used at a 1:1000 dilution. Monoclonal antibody against the EGF receptor (α-EGFR, Transduction Laboratories) and one autophosphorylation site from the EGF receptor (Ty-ro-1063) and 10 mM ATP in HNTG buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM NaVO₄, 100 μM phenylmethylsulfonyl fluoride, pH 7.4), sonicated two times for 10 s, and clarified. Protein concentrations were determined for the Bio-Rad DC protein assay.

For use in immunofluorescence studies, A431 cells were grown to 50–70% confluence on coverslips in six-well tissue culture plates. They were then washed twice with PBS, serum-starved for 16 h in OPTI-MEM (Life Technologies), and left untreated or given 20 ng/ml EGF for 4 h at 4 °C with 20–200 ng/ml EGF. This response was maintained over a 105 range (20–200 ng/ml) at a final concentration of 91 pmol of GST-EGFR-(987–1028) reagent was then plotted on the standard curve to determine phosphorylation stoichiometry of the EGF receptor, we generated the GST-EGFR-(987–1028) reagent, which could be used to quantitate both the number of phosphates and the number of receptors via Western blot analysis. This protein was phosphorylated using an in vitro phosphorylation reaction with the EGF receptor. A431 cells treated with 100 ng/ml EGF for 5 min were centrifuged in HNTG, and the supernatant was incubated with a monoclonal antibody against the EGF receptor (Promega). GST-EGFR-(987–1028) was added to the pellet at a final concentration of 91 μM in kinase reaction buffer (10 mM MnCl₂ and 50 μM ATP in HNTG buffer), and the reaction proceeded for 15 min at 4 °C with rocking. After the addition of 1 ml of HNTG, the reaction was centrifuged, and the supernatant was removed and immunoprecipitated overnight with a monoclonal antibody against phosphotyrosine to ensure that only phosphorylated GST-EGFR-(987–1028) was used in the experiment. Control experiments showed that GST alone was not phosphorylated in this system. To determine the concentration of the phosphorylated GST-EGFR-(987–1028), 10 μM of the immunoprecipitate and serial dilutions of a GST fusion protein of known concentration were subjected to Western blot analysis with α-GST and analyzed on a Phosphor Imager. The signals from the serially diluted standard were graphed, and a curve was determined by linear regression. The signal from the EGFR-(987–1028) reagent was then plotted on the standard curve to determine the concentration.

Duplicate SDS-PAGE gels were prepared containing known amounts of GST-EGFR-(987–1028) and the α-Act. EGFR and PLC-SH2 precipitations from 500 μg of the lysate of A431 cells that were serum-starved and either untreated or treated with 20 ng/ml EGF for 1, 30, and 60 min. The gels were transferred to nitrocellulose, and the filters were blotted with α-EGFR and α-Tyr(P). PhosphorImager analysis was performed to identify the amount of receptor and phosphotyrosine in each precipitation using the signals from EGFR-(987–1028) to determine a signal/molecule ratio. The experiment was performed three times, and Fig. 6 represents the average of these three experiments.

RESULTS

PLC-SH2 Identifies Temporal Differences in EGF Receptor Activation—During studies on the activation kinetics of the cells treated with biotin-EGF were incubated with PLC-SH2, washed, and then incubated with FITC conjugated to avidin (Boehringer Mannheim) used at 1:200 dilution. All incubations were for 30 min (except permeabilization) and were performed in the dark at 4 °C. Both primary reagents were incubated at the same time, and separate experiments confirmed that there was no competition between these two reagents for binding to the EGF receptor. Incubation with GST protein alone (10 μg/ml), followed by α-GST at a 1:100 dilution and FITC-conjugated goat anti-mouse antibody at a 1:20 dilution showed no binding. The coverslips were mounted onto slides with SLOW-FADE (Molecular Probes, Inc.) and sealed with nail polish. Microscopy was performed using a Zeiss Axiosvert 100 confocal microscope with a Bio-Rad MRC 600 krypton-argon laser and was amplified at 15 milliwatts. The cells were viewed at a magnification of ×63 using optical sections of 0.25-μm thickness. The excitation and emission wavelengths were 488 and 520 nm for FITC and 647 and 667 nm for Cy3, respectively.

Pretreatment of the EGF Receptor—For the PLC-SH2 precipitations, 500–4000 μg of fresh A431 cell lysate, untreated or treated with 20 ng/ml EGF, was incubated for 4 h at 4 °C with 10–200 μg of PLC-SH2 or 10 μg of GST protein alone prebound to 50–300 μl of glutathione-Sepharose (Pharmacia) in TGG buffer supplemented with dithiothreitol at 5 mM for 1 h. At 4 °C. The pellets were washed three times with TGG and resuspended in the same buffer. For the co-precipitation experiments, the pellets were three times with HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and 5 mM dithiothreitol at 4 °C with a 5-min rocking period between washes at 4 °C. For antibody immunoprecipitations, 150–400 μg of A431 cell lysate was incubated with 10–50 μg of a sheep polyclonal antibody against the EGF receptor (Life Technologies), 10 μg of a monoclonal antibody against phosphotyrosine (Transduction Laboratories), 5 μg of α-Act. EGFR, or 10 μg of a monoclonal antibody against phospholipase C-γ1, or 25–75 μl of protein G + A-agarose (Oncogene Science) and 10–20 μl of formalin-fixed Staph A cells (Life Technologies) with the same buffers and as above. For the PLC-SH2 characterization and stoichiometry experiments, equal amounts of pellets were run on SDS-PAGE. In the co-precipitation experiments, the amount of pellet sample run on SDS-PAGE was adjusted to achieve equal α-EGFR signals between the pellets and the receptor.

Phosphotyrosine Stoichiometry—To determine phosphorylation stoichiometry of the EGF receptor, we generated the GST-EGFR-(987–1028) reagent, which could be used to quantitate both the number of phosphates and the number of receptors via Western blot analysis. This protein was phosphorylated using an in vitro phosphorylation reaction with the EGF receptor. A431 cells treated with 100 ng/ml EGF for 5 min were washed in HNTG, and the supernatant was incubated with a monoclonal antibody against the EGF receptor (Promega). GST-EGFR-(987–1028) was added to the pellet at a final concentration of 91 μM in kinase reaction buffer (10 mM MnCl₂ and 50 μM ATP in HNTG buffer), and the reaction proceeded for 15 min at 4 °C with rocking. After the addition of 1 ml of HNTG, the reaction was centrifuged, and the supernatant was removed and immunoprecipitated overnight with a monoclonal antibody against phosphotyrosine to ensure that only phosphorylated GST-EGFR-(987–1028) was used in the experiment. Control experiments showed that GST alone was not phosphorylated in this system. To determine the concentration of the phosphorylated GST-EGFR-(987–1028), 10 μM of the immunoprecipitate and serial dilutions of a GST fusion protein of known concentration were subjected to Western blot analysis with α-GST and analyzed on a Phosphor Imager. The signals from the serially diluted standard were graphed, and a curve was determined by linear regression. The signal from the EGFR-(987–1028) reagent was then plotted on the standard curve to determine the concentration.
EGF receptor, far Western blot analysis with the SH2 domains of PLC-γ₁ revealed an interesting pattern (Fig. 1A). Quantitation of the receptor signal and normalization to the 1-min time point showed that the maximum signal occurred at 60 min, with a 2-fold increase over time (Fig. 1B). In contrast, it has been shown that maximal receptor autophosphorylation occurs within 1–5 min, and this level remains relatively constant for up to 1 h (2, 25). Therefore, it was possible that we had identified a subset of receptors with activation and phosphorylation kinetics different from the general pool of active receptors. One potential explanation for our observation may be simply due to differences in the reagents used. The previous studies used anti-phosphotyrosine antibodies to identify active EGF receptors and hence reflect the amount of phosphotyrosine at each time point, not the amount of active receptor. PLC-SH2 recognizes only a single autophosphorylation site on the EGF receptor (tyrosine 992) and thus directly represents a portion of active receptor. To determine if our observation was indeed due simply to valence differences of the reagents, we compared PLC-SH2 blots with an antibody that recognizes activated EGF receptors (Transduction Laboratories; Ref. 25), which we call α-Act. EGFR.

α-Act—EGFR is a monoclonal antibody that recognizes the ligand-activated and phosphorylated form of the EGF receptor but does not recognize other tyrosine-phosphorylated proteins. It recognizes an EGF receptor mutant that lacks Tyr₁⁰⁶⁸, Tyr₁¹⁴⁸, and Tyr₁¹⁷³ and is not inhibited by 0.1 mM phosphoserine, phosphothreonine, or phosphotyrosine; thus, it is an antibody that recognizes an active conformation rather than phosphotyrosine (25). This antibody can recognize the EGF receptor in non-denaturing and denaturing conditions, and cleavage studies have shown that the epitope for this antibody is on the carboxyl terminus of the receptor distal to amino acid 1052 (25). Competition experiments using a 100-fold molar excess of PLC-SH2 failed to affect binding of this antibody to EGF receptors in Western blots and immunocytochemistry, which confirmed that PLC-SH2 and α-Act. EGFR recognize distinct epitopes.

Blotting with α-Act—EGFR revealed an increase of 1.4-fold from 1 to 5 min, and, similar to studies using anti-phosphotyrosine antibodies, the signal remained essentially the same until 60 min and then decreased by 120 min (Fig. 1B), suggesting that the differences observed with PLC-SH2 were not merely due to differences in reagents used.

Specificity of PLC-SH2—Having observed these initial differences, we wished to confirm the specificity of this reagent. Lysates from A431 cells untreated or stimulated with EGF for 5 min were used for immunoprecipitation with an antibody to the EGF receptor (Fig. 2A). Pellets were subjected to Western blot analysis with an antibody against the EGF receptor (α-EGFR) or PLC-SH2, which confirmed that PLC-SH2 recognized the EGF receptor and required EGF-induced activation for its recognition. Next, we determined if the PLC-SH2 reagent recognized other tyrosine-phosphorylated proteins. Total cell lysate was examined by Western blot analysis with α-Tyr(P) and PLC-SH2. While α-Tyr(P) recognized numerous bands in addition to the EGF receptor in stimulated cells, the PLC-SH2 reagent recognized only the EGF receptor (Fig. 2B). To verify that PLC-SH2 recognized phosphorylated EGF receptors in their native form, and only Tyr(P)⁹⁹² receptors, a greater than 100-fold molar excess of PLC-SH2 was used to precipitate the EGF receptor from EGF-stimulated A431 cells (Fig. 2C). Western blot analysis of the supernatant and pellet with α-EGFR demonstrated that only 15–20% of the phosphorylated receptors were precipitated under conditions in which all of the PLC-SH2 was precipitated. This is in agreement with the fraction of EGF receptor that has been reported to be phosphorylated on this site (9, 10). An α-Tyr(P) immunoprecipitation blotted with α-EGFR and PLC-SH2 showed that the decrease in mobility of the EGF receptor band in the PLC-SH2 precipitation pellet lanes was due to phosphorylation of the receptor. Immunoprecipitation of lysates from EGF-stimulated A431 cells with a monoclonal antibody against PLC-γ₁ showed that, under conditions in which all of the native PLC-γ₁ is precipitated, only a very small fraction of the total EGF receptor pool was co-precipitated (Fig. 2D).

Differential Trafficking of the Tyr(P)⁹⁹² Subset—After ligand binding, most EGF receptors are internalized in clathrin-coated pits within 1–10 min and then transit through the endosomal system (2, 4–6, 12, 15, 17, 26, 27). By 5–15 min, the majority of internalized receptors are concentrated in the juxtanuclear area in multivesicular bodies, where they enter into lysosomes and are degraded. The temporal differences identified in the first experiment prompted us to investigate any differences in the endocytosis of EGF receptors. PLC-SH2 and

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2 D. R. Emlet and A. J. Wong, unpublished observations.
Subsets of EGF Receptors

Both reagents strongly stained cell surface membrane ruffles 1 min after the addition of EGF. However, the cell membrane did not always uniformly label with the PLC-SH2 reagent in comparison with α-Act. EGF receptor (3, 1 min.). Punctate staining within the cytoplasm corresponding to early endosomes was seen only with α-Act. EGF receptor, a difference that persisted after 5 min of EGF exposure. At 10 min, PLC-SH2 did show punctate staining in most cells. A major difference was that juxtanuclear structures could be detected with α-Act. EGF receptor, but despite the size and extent of these bodies there was no staining by PLC-SH2. Fig. 4A shows double labeled micrographs of one cell displaying this phenomenon. The top micrograph shows the signal from the α-Act. EGF receptor antibody, and the arrows indicate the presence of large, juxtanuclear structures stained with this reagent. The bottom micrograph shows the signal from PLC-SH2. While punctate staining corresponding to early endosomes can readily be seen, there is no staining of the juxtanuclear structures. After 15 min, more extensive punctate staining was seen with PLC-SH2 as well as labeling of the juxtanuclear structures. However, while these structures were uniformly labeled by α-Act. EGF receptor, only distinct portions were labeled by PLC-SH2, suggesting that vesicles containing PLC-SH2-labeled receptors had fused separately to this putative multivesicular body (Figs. 3, 15 min., inset). Greater co-localization within these bodies was noted at 30 min, although it was possible in a few cells to identify juxtanuclear structures that were stained by PLC-SH2 but not by the antibody against the activated EGF receptor. This observation may reflect the fact that at this time point a greater proportion of endocytosed receptors are stained by PLC-SH2. After 60 min, no differences in the localization of the two reagents could be found. To confirm these findings, experiments were also performed using EGF conjugated to biotin as the reagent for detecting activated receptors, which produced a staining pattern similar to that of α-Act. EGF receptor. Additionally, double labeling occasionally revealed the presence of cells that stained very weakly or not at all with the SH2 domain reagent yet were avidly stained with α-Act. EGF receptor. Fig. 4B shows micrographs of a cell that exhibits this staining pattern. The top micrograph shows staining with α-Act. EGF receptor, and both plasma membrane and juxtanuclear structure staining can readily be seen. The bottom micrograph shows staining of the same cell with PLC-SH2 at the same laser intensity. Despite the fact that adjacent cells show staining, the cell identified by the arrow is devoid of any staining by this reagent.

Differential Association of SH2 Proteins with the Tyr(P)992 Subset—We wondered if any differences existed in the binding of SH2 proteins to the general population of EGF receptors versus the Tyr(P)992 subset. Lysates from A431 cells treated with EGF for 30 min were precipitated with either an antibody against the EGF receptor or PLC-SH2, and the supernatants (S) and pellets (P) were used for Western blot analysis with antibodies to various EGF receptor-associating SH2 proteins (Fig. 5). Preliminary experiments were performed so that equal amounts of EGF receptor were loaded in the pellet lanes. This showed that EGF receptors precipitated with PLC-SH2 showed an enhanced association with Ras-GAP, PI 3-kinase, and SHPTP2/syp. In contrast, the α-EGFR-precipitated receptors exhibited a 1.5-fold greater ability to co-precipitate Grb2 than the PLC-SH2 precipitated receptors. Interestingly, although SOS indirectly associates with the EGF receptor through Grb2, these Tyr(P)992 receptors were still capable of co-precipitating a greater amount of SOS. These results show that differences can exist in the association of SH2 proteins with the Tyr(P)992 α-Act. EGF receptor were used to label EGF receptors in A431 cells during the process of activation and internalization.

**Fig. 2.** Binding characteristics of PLC-SH2. A, lysates from A431 cells either not treated (−) or treated for 5 min with 20 ng/ml EGF (+) were immunoprecipitated (IP) with an antibody against the EGF receptor (Life Technologies), and the pellets were used for Western blot analysis with an antibody to the EGF receptor (α-EGFR) and PLC-SH2. B, the same cell lysates as in A were blotted with either an antibody against phosphotyrosine (α-pTyr) or PLC-SH2. C, lysates from A431 cells treated with EGF for 5 min were precipitated with GST alone, PLC-SH2, or an antibody against phosphotyrosine. Blots containing equal volumes of the supernatants (S), and pellets (P), were incubated with α-EGFR, PLC-SH2, or an antibody against GST (α-GST). The size of molecular weight standards is shown on the left. D, EGF-treated lysates were immunoprecipitated with an antibody against PLC-γ (α-PLCγ), and equal amounts of the supernatant (S) and pellet (P) were used for blotting with α-EGFR and α-PLCγ. The size of molecular weight standards is shown on the right.
population versus the general population of EGF receptors. The fact that greater amounts of SH2 proteins and SOS associate with the Tyr(P)992 population suggests that this subset may play a significant role in signaling.

**Phosphotyrosine Stoichiometry of the Tyr(P)992 EGFR Subset**—Our previous results suggested that there might be a difference in the stoichiometry of tyrosine phosphorylation of Tyr(P)992 receptors. To examine this directly, lysates from A431 cells that were serum-starved and either untreated or treated with EGF for 1, 30, and 60 min were precipitated with α-Act. EGFR or PLC-SH2, and the pellets were subjected to Western blot analysis. These blots also contained a GST fusion protein encoding the epitopes for α-EGFR and a single tyrosine phosphorylation site (GST-EGFR-(987–1028)). Thus, following a kinase reaction and anti-phosphotyrosine purification (see “Experimental Procedures”), this protein would provide a known standard for both tyrosine phosphorylation and EGFR receptor content. This analysis showed that the number of phosphotyrosines per receptor for both α-Act. EGFR and PLC-SH2 precipitations remained constant over the period of EGF treatment (Fig. 6). For both the α-Act. EGFR and PLC-SH2 precipitations, there was a low, basal level of phosphorylation in the unstimulated lysates. Following EGF treatment, the ratio for α-Act. EGFR was approximately 1 phosphotyrosine/molecule from 1 to 60 min of EGF treatment. Interestingly, we found that the Tyr(P)992 subset was more heavily phosphorylated, with approximately 2 phosphates/molecule from 1 to 60 min of EGF treatment, demonstrating yet another difference in this population of receptors. These results suggest that the basis for the enhanced SH2 protein association with this population of receptors was a higher degree of phosphorylation.

**FIG. 3.** Double-labeling studies on A431 cells demonstrate differential trafficking of the EGF receptor. A431 cells were grown on coverslips, serum-starved for 16 h, and left untreated or treated with EGF for the times indicated and then incubated with Cy5-conjugated PLC-SH2 and α-Act. EGFR followed by FITC-conjugated anti-mouse antibody to identify α-Act. EGFR. The cells were then analyzed by confocal microscopy. The numbers above correspond to the time of EGF treatment, and the reagents for each row are shown on the left. 0 min., control showing no staining by either reagent in absence of EGF. 1 min., cell membrane labeling by the PLC-SH2 reagent is not uniform, since surface microvilli (large arrow) are stained by both reagents but internal membranes are not (arrowhead). Small punctate vesicles are also seen with α-Act. EGFR (arrow) but are not detected with PLC-SH2. 5 min., intracellular punctate staining by PLC-SH2 is still not prominent. 10 min., punctate staining by PLC-SH2 can now be seen in most cells. 15 min., co-localization in juxtanuclear bodies is seen with both reagents (arrows), but enlargement in the inset shows only partial labeling of these structures by PLC-SH2. 30 min., coincident labeling by both reagents is greater, although it is possible to detect juxtanuclear structures that stain only with PLC-SH2 (arrow). 60 min., staining by both reagents shows extensive co-localization. Each micrograph corresponds to a 0.25-μm horizontal section through the cells. Bar, 10 μm.

**FIG. 4.** Confocal microscopy with PLC-SH2 can identify large differences in receptor processing. A, enlargement of cell treated with EGF for 10 min to show lack of staining in multivesicular structures (arrow) by the PLC-SH2 reagent. B, cells treated with EGF for 15 min, showing an individual cell (arrow) that stains well with α-Act. EGFR but not with PLC-SH2. Each micrograph corresponds to a 0.25-μm horizontal section through the cells. Bar, 10 μm.
Tyr(P)992 receptors as opposed to receptors that cannot bind to PLC-SH2 detects the general population of PLC-active receptors are summarized in Fig. 7. Overall, it was suggested that PLC-SH2 binds to Tyr(P)992 receptors with a higher affinity than to other receptors, possibly explaining the slower rate of endocytosis and the higher level of phosphorylation observed for this subset of receptors.

One potential concern in this study was that endogenous PLC-γ1 could be affecting the activity of PLC-SH2 in vitro. This was highly unlikely for a number of reasons. Studies on SH2 domain affinities have shown that along with a very high rate of association, there is also a high rate of dissociation, which causes a rapid exchange of proteins on the receptor (28). Hence, each receptor can interact with a succession of SH2 proteins, which makes it feasible for exogenous SH2 domains to bind receptors as the endogenous SH2 proteins turn over. Second, and as a possible consequence of this rapid turnover, there is a low fraction of proteins bound to the receptor at any time (Ref. 29, Fig. 2D), despite the high EGFR expression level in A431 cells. To support this, staining with α-PLCγ1 antibody confirmed that there was no formation of vesicles or juxtanuclear bodies with time as assayed by confocal microscopy.

Finally, there is a 20-fold molar excess of receptor capable of binding to PLC-γ1 per cell (based on ~2 × 10^5 Tyr(P)992 receptors and ~10^4 copies of PLC-γ1 (30)). Taken together, these facts indicate that PLC-SH2 detects the general population of Tyr(P)992 receptors as opposed to receptors that cannot bind PLC-γ1.

We have found two distinct properties of the Tyr(P)992 EGFR receptors during signaling in A431 cells. One property is a slower rate of endocytosis. Since the autophosphorylation sites of the EGFR receptor are oriented toward the cytoplasm during endocytosis (2) and the receptor remains active during this time (16), it is capable of transmitting signals until it is degraded. The rate of internalization will affect signaling by affecting the duration of interaction with effector molecules. While Tyr(P)992 EGFR receptors constitute only 15–20% of all active receptors, a substantial amount of the enzyme can be activated due to the slower rate of internalization. This would explain other experiments, which showed that despite the majority of EGFR receptor being internalized within 30 min, the activation of PLC-γ1 increased linearly up to 60 min following EGFR stimulation (31), which was paralleled by an increase in tyrosine phosphorylation of PLC-γ1 (32) and the association of phosphorylated PLC-γ1 with the cytoskeleton (33).

A second characteristic of the Tyr(P)992 receptor subset in A431 cells is a higher stoichiometry of phosphorylation and greater binding of SOS, Ras-GAP, PI3-kinase, and SHP2p56. This would suggest that, despite being a small percentage of the active receptor pool, this subset of receptors may have a greater role in linking the EGF receptor to a diversity of downstream signaling pathways. Of particular interest was the observation that greater amounts of SOS were associated with Tyr(P)992 receptors, although this population bound relatively less Grb2. SOS is a guanine nucleotide exchange factor for Ras, and the formation of a complex with the EGFR receptor and Grb2 stimulates Ras activation (34–36). We speculate that Ras activation is preferentially associated with these receptors. Recently, it has been shown that the EGFR receptor and other signal transduction proteins can be segregated into specific intracellular compartments, and our results could provide further clues into how these proteins are sorted. Di Guglielmo et al. (37) found greater amounts of SHC, Grb2, and SOS with endosomal exosomes as opposed to plasma membrane EGFR receptors. They also concluded that receptor internalization can serve as a mechanism to prolong signaling. It would be of interest to determine if Tyr(P)992 receptors are the subset that associates with SOS in endosomes.

Evidence that SH2 domain-containing proteins affect receptor processing is beginning to surface. The high affinity binding
sites for PI 3-kinase on the platelet-derived growth factor receptor are necessary in the early steps of endocytic trafficking (38), and PI 3-kinase catalytic activity is required to divert the receptor to a degradative pathway (39). Furthermore, it has been shown that disruption of Grb2 binding to the EGF receptor prevents the endocytosis of that receptor (40). The results presented here suggest that some protein that binds to the Tyr(P)992 receptor subclass, perhaps PLC-1 itself or PI 3-kinase, may be involved in distinguishing the intracellular routing of the EGF receptor. Collectively, the findings presented here and those of Joly et al. (38, 39), Sorkin et al. (13), Helin et al. (12), and Wang and Moran (40) show that autophosphorylation sites are essential for proper internalization and, moreover, that an individual site may determine specific routing of the receptor. It is possible that RPTKs undergo continuous phosphorylation/dephosphorylation, and this also contributes to the pattern of internalization. However, the fact that the number of Tyr(P)992 receptors remains constant over time (Fig. 6), suggests that this phenomenon does not play a large role in A431 cells. Regardless, this phenomenon would not alter our conclusions about the role of autophosphorylation sites in the differential trafficking of EGF receptors. It is important to keep in mind that these characteristics have been identified in A431 cells that overexpress the EGF receptor and thus may not be extrapolative to cells with normal levels of receptors. Present evidence indicates, however, that some of these findings are also true in other cells.

Using other SH2 domains with the methods described might provide further insights into receptor processing and signaling. For example, another SH2 domain may define the population of receptors that rapidly undergoes endocytosis and degradation. One implication would be that the signals from the SH2 proteins that bind this autophosphorylation site would be rapidly induced and terminated. Employing SH2 domains from proteins involved in activating Ras, such as Grb2 or SHC, may reveal new aspects of how oncogenic signals are transmitted from the EGF receptor. Undoubtedly, there are a wide variety of other RPTKs and physiologic situations that could be studied using SH2 domains as phosphotyrosine site-specific reagents.

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Fig. 7. A model for tyrosine phosphorylation status specificity during the internalization of EGF receptors in A431 cells. This schematic summarizes the differences in the Tyr(P)992 subset of EGF receptors. Upon the addition of EGF, active but non-Tyr(P)992 receptors identified by α-Act. EGFR (white rectangles) immediately begin to internalize into endosomes, whereas some Tyr(P)992 receptors identified by PLC-SH2 (gray rectangles) become activated but do not internalize. Within 5–15 min, non-Tyr(P)992 receptors have entered into multivesicular bodies, while the Tyr(P)992 receptors increase in number and can be found in endosomes. 15–30 min after the EGF addition, Tyr(P)992 receptors continue to increase in number, and vesicles containing these receptors begin to fuse with the multivesicular bodies already containing the non-Tyr(P)992 receptors. By 60 min of EGF treatment, the number of Tyr(P)992 receptors has maximized, and there are equal numbers of non-Tyr(P)992 and Tyr(P)992 receptors within the multivesicular bodies. From 1 to 60 min of EGF treatment, non-Tyr(P)992 receptors contain approximately 1 phosphotyrosine/molecule, while Tyr(P)992 receptors contain approximately 2 phosphotyrosines/molecule.
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