The epidermal growth factor receptor (EGFR) is activated by ionizing radiation (IR) in many human carcinomas, mediating a cytoprotective response and subsequent radioresistance. The underlying molecular mechanisms remain to be understood, and we propose here a specific role for the Tyr-992 residue of EGFR and examine its regulation by the phosphatase, SHP2. The -fold increase in phosphorylation of Tyr-992 in response to IR is twice that seen with ligand (EGF) binding. Mutation of Tyr-992 blocked completely IR-induced EGFR phosphorylation and reduced activation of the downstream signaling molecule, phospholipase Cγ. IR has previously been demonstrated to inhibit activity of protein-tyrosine phosphatases. Following protein-tyrosine phosphatase inhibition by sodium vanadate both EGFR expressing Chinese hamster ovary (CHO) and A431 exhibited up to an -fold increase in the basal level of Tyr-992 phosphorylation, significantly higher than that seen with Tyr-1173, Tyr-1068, and total EGFR Tyr. CHO cells expressing a SHP2 mutant also demonstrated up to an -fold increase in the basal level of Tyr-992 phosphorylation. In this study we show the unique association of SHP2 with EGFR in response to IR, with up to a -fold increase in the direct association of endogenous SHP2 with EGFR-wt in response to 2 gray of IR in both CHO and A431 cells. Mutation of Tyr-992 abolished this response. In conclusion we have identified several differentially activated Tyr residues, one of which is not only more sensitive to activation by IR, translating into differential activation of downstream signaling, but uniquely modulated by the phosphatase SHP2.

We, and others have shown EGFR to be activated by ionizing radiation (IR) resulting in cytoprotective downstream signaling primarily through the mitogen-activated protein kinase (MAPK) cascade, thus enhancing sensitivities of cancer cells to the toxic effects of IR (2). Overexpression of a dominant-negative EGFR mutant, EGFR-CD533, disrupts the cytoprotective response by preventing radiation-induced activation of the receptor and its downstream effectors, conferring radiosensitization of tumor cells (3). Although EGFR has been established as an important molecular target and prognostic marker in cancer therapy, the initial molecular events involved in EGFR activation by radiation are not well understood.

The general phosphorylation of EGFR Tyr residues by radiation is a well established phenomenon (2, 3), but there are currently no reports showing the effects of radiation on any specific EGFR Tyr phosphorylation sites. Because the individual Tyr sites demonstrate differing affinities for the many adaptor molecules involved in downstream signaling from EGFR we are particularly interested in which of these sites respond specifically to IR rather than the ligand EGF. Thus, we have addressed the molecular mechanisms of EGFR activation by examining IR- and EGF-induced phosphorylation of specific EGFR Tyr residues in A431 squamous carcinoma cells and EGFR expressing CHO cells.

The EGFR autophosphorylation sites Tyr-992 and Tyr-1173 have been shown to play a critical role in the activation of the MAPK cascade following EGF stimulation. Tyr-992 is a high-affinity binding site for phospholipase Cγ (PLCγ), and is required for PLCγ activation by EGF (4, 5). We have also shown radiation-induced changes in free cytosolic (Ca^{2+}) to be dependent on PLCγ (2, 6, 7). PLCγ activates the Ras/MAPK cascade through inositol 1,4,5-triphosphate production and oscillations in cytosolic (Ca^{2+}) (1, 7, 8). Tyr-1173 serves as a major binding site for Shc, an adaptor protein involved in signaling between EGFR and Ras (9, 10). In addition, Tyr-992 and Tyr-1173 are minor binding sites for Shc and PLCγ, respectively (10, 11). Thus, these two Tyr residues are potentially involved in MAPK activation. In this study we have tested the role of the EGFR in radiation-induced PLCγ and Shc phosphorylation using the specific EGFR kinase inhibitor, AG1478. We also evaluated the role of Tyr-992 and Tyr-1173 in these responses, using EGFR constructs specifically mutated (Tyr-Phe) in these residues.

Tyr-992 and Tyr-1173 have also been demonstrated to be binding sites for phosphatases (12, 13), which play an important role in Tyr dephosphorylation and hence modulation of EGFR activity. In studies on the radiation-induced radicals on phosphatases, we previously measured radiation-induced reactive oxygen/nitrogen species (ROS/RNS) at the single cell level by fluorescence microscopy (14–16). ROS/RNS generation oc-
curred within 2–5 seconds of radiation exposure (1–10 Gy) and persisted for 2–5 min after irradiation. ROS species, such as H₂O₂, have been shown to induce phosphorylation of the EGFR (4, 5) and because H₂O₂ also causes inhibition of protein-tyrosine phosphatase (PTP) activity (16, 17). Sequence specificity in recognition of the EGFR has been shown for PTP1B and SHP2 at Tyr-992 (13, 21, 22), and SHP-1 has been demonstrated to bind to the EGFR at Tyr-1173 (23). The modulation of EGFR phosphorylation by both of these phosphatases, however, has only been demonstrated in response to ligand binding. In this study we examined the modulation of EGFR by phosphatases, including the Tyr-992-specific phosphatase, SHP2. We also studied the interaction of SHP2 and EGFR in response to IR in both A431 and CHO cells expressing either wild type EGFR or its Tyr-992 mutation.

### MATERIALS AND METHODS

**Reagents—** Unless specified otherwise, all reagents were obtained from Sigma. RPMI 1640 medium and Lipopolysaccharide (LPS) reagent were obtained from Invitrogen; fetal bovine serum was purchased from HyClone (Logan, UT). AG1478 was obtained from Calbiochem (La Jolla, CA). Protein G Plus/Protein A–agarose beads were purchased from Oncogene Research Products (San Diego, CA). Antibodies phospho-PLC-y1 (Tyr-783), phospho-Ick (Tyr-317), phospho-Src (Tyr-416), total Tyr(P) (T02), EGFR Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, and horse radish peroxidase (HRP)-linked secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase-conjugated antibodies were purchased from Vector Laboratories (Burlington, CA). EGFR Ab-15 (for immunoprecipitation) and EGFR Ab-14 (for Westerns) are purchased from NeoMarkers (Fremont, CA). PLC-y1, Src, and SHP2 from Santa Cruz Biotechnology (Santa Cruz, CA). Chinese hamster ovary (CHO-K1) and A431 human squamous carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA).

**Expression Constructs and Adenovirus—** Wild-type EGFR and Y992F/Y1173F mutant EGFR constructs were kindly donated by Dr. Axel Ulrich (Max-Planck Institute, Martinsried, Germany). Wild type and dominant negative (c/s) SHP2 constructs were kindly donated by Dr. Jeffrey Pessin (SUNY at Stony Brook, NY), as previously described (24). These same constructs were subcloned into adenovirus (25).

**Cell Culture and EGFR Treatment—** Culture of A431 cells has been previously described (2). A431 cells were plated at 4.25 × 10⁵ cells/6-cm dish and transfected with the appropriate DNA described below. Following a 24-h recovery period, cells were starved for 16–18 h prior to IR or EGFR treatment.

**Transfection—** CHO cells were plated at 6 × 10⁵ cells/6-cm dish and transfected 24 h later with 1 µg/6-cm dish of DNA (EGFR-wt, Y992F, or -Y1173F mutants) using Lipofectamine PLUS (according to manufacturer instructions). For SHP2 experiments cells were transfected (as described previously) with 0.5 µg of EGFR or Y992F mutant EGFR plus either carrier DNA, wt-SHP2 or c/s SHP2. Cells were lysed 24 and 48 h following transfection for Western blot analyses.

**Adenoviral Infection—** A431 cells were plated at 4.25 × 10⁵ cells/6-cm dish and cultured at 37 °C for 2 days. Cells were exposed for 1 h to adenovirus containing LacZ, wild type SHP2, or a dominant negative c/s SHP2. The three viruses were tested at multiplicity of infections of 5, 10, and 50. Cells were lysed for Western blot analyses 24 and 48 h following infection.

**Cell Lysis and Western Blotting—** Cells were rinsed with ice-cold phosphate-buffered saline and snap-frozen on dry ice at the appropriate times after treatment. Cells were scrapped into a lysis buffer (25 mM Tris, pH 7.4, 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.5 mM EDTA, 5% glycerol, 1% Triton-X) containing protease inhibitors (1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 100 µg/ml phenylmethylsulfonyl fluoride) and passed 5 times through a 20-gauge needle and syringe. Samples were centrifuged at 11,000 × g for 15 min at 4 °C, and supernatant protein concentrations were determined by the Bradford assay. For whole cell lysates, 5 × loading buffer (50 mM NaPO₄, 5% SDS, 0.25% bromphenol blue, 12.5% 2-mercaptoethanol, and 10% glycerol) was added to lysates to achieve 1× concentration; samples were then boiled for 5 min prior to Western blotting. Equal amounts of protein were fractionated on SDS, 6% polyacrylamide gels and protein was transferred electrophoretically onto nitrocellulose membranes. Membranes were probed with the appropriate primary and secondary antibodies. Blots were analyzed by chemiluminescence detection and densitometry. With the exception of Total Tyr(P) (1:2000) and SHP2 (1:2500), all antibodies were used at a dilution of 1:1000 in 5% milk, Tris-buffered saline-Tween. The horseradish peroxidase-conjugated anti-rabbit antibody was diluted 1:2000 and the alkaline phosphatase-conjugated antigoat antibody was diluted 1:7500, also in 5% milk, Tris-buffered saline-Tween. Blots were stripped with a 62.5 mM Tris (pH 7.4), 2% SDS, 0.7% 2-mercaptoethanol solution at 55 °C for 30 min, and re-probed as described.

**Immunoprecipitation—** Cells were lysed on ice immediately following treatment. 4 µg of anti-EGFR (Ab-15), ERBB2 (Ab-11), or 2 µg of anti-SHP2 antibody was added to 400 µg of lysate in a total volume of 500 µl. After mixing for 2 h at 4 °C, 30 µl of protein A/G-agarose beads were added to each lysate and mixed for 1 h at 4 °C. beads were rinsed three times with lysis buffer (plus inhibitors). 30 µl of 2× loading buffer was added to each sample before boiling for 10 min.

## RESULTS

**Ionomizing Radiation and EGF Differentially Phosphorylate Specific Tyr Residues within EGFR—** We have previously shown that radiation activates the EGFR by general phosphorylation of certain Tyr residues, and that inhibition of EGFR-phospho-Tyr results in radiosensitization (2, 3). The purpose of this study was to examine specific EGFR Tyr residues with reported links to MAPK cascade activation, and their potential roles in radiation-induced EGFR activation and downstream signaling. To focus on individual phosphorylation sites, phosho-specific antibodies were used against various Tyr residues within the cytoplasmic domain of the EGFR known to be involved in the binding of adapter proteins involved in downstream signaling.

Examination of the phosphorylation pattern of individual Tyr residues identified three distinct categories. The first consisted of residues unaffected by either IR or EGF. These residues, e.g. Tyr-1068 and Tyr-1086, exhibited a relatively high basal level of phosphorylation that remained unchanged after exposure to 2 Gy of IR or 10 ng/ml EGF (Fig. 1A; Table I).

The second category consisted of Tyr residues more highly phosphorylated by EGF than IR, paralleling the Tyr phosphorylation response of the EGFR as a whole. This included Tyr-845, Tyr-1045, and Tyr-1173. Tyr-845 demonstrated a 11.6-fold increase in phosphorylation compared with the untreated control by 2 Gy IR in A431 human carcinoma cells (Fig. 2). The purpose of this study was to examine specific EGFR Tyr residues with reported links to MAPK cascade activation, and their potential roles in radiation-induced EGFR activation and downstream signaling. To focus on individual phosphorylation sites, phosho-specific antibodies were used against various Tyr residues within the cytoplasmic domain of the EGFR known to be involved in the binding of adapter proteins involved in downstream signaling.

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and decreased over the subsequent 8 min. IR induced an almost 6-fold increase in Tyr-992 phosphorylation over that seen in the untreated control and similar to total Tyr(P) of EGFR, phosphorylation peaked at 2 min following IR exposure and decreased over the next 8 min. Exposure to concentrations of EGF (10 ng/ml) that provide comparable changes in EGFR phosphorylation increased total Tyr(P) by up to 6-fold peaking at 5 min, but Tyr(P)-992 was only increased 3-fold (Fig. 2). The kinetics of ligand-induced phosphorylation of Tyr-992 were similar to those of total EGFR phosphorylation, the effect of radiation on Tyr-992 relative to Tyr-1173 was not as pronounced in CHO cells as in A431 cells.

**EGFR Dependence of Radiation-induced PLCγ and Shc Phosphorylation in A431 Cells**—Because PLCγ and Shc have been shown to bind to Tyr-992 and Tyr-1173 with subsequent activation of the MAPK cascade (4, 5, 7–10), we tested whether the radiation-induced phosphorylation of these effectors was dependent upon EGFR. In addition, phosphorylation of Src was examined, because Src-mediated phosphorylation of the EGFR at Tyr-845 has been shown to modulate receptor function (26). Previous studies have shown that critical phosphorylation sites for activation of PLCγ1 and Shc are Tyr-783 and Tyr-917, respectively (27, 28); thus, the phosphorylation of these particular sites was examined.

Using the same time and dose strategies as before, a mean 6.2-fold induction of PLCγ phosphorylation was observed following radiation treatment (Fig. 4). Increases in phosphorylation of Shc family members were observed following irradiation; the mean fold changes (n = 3) were 2.3, 3.0, and 3.5 for the 66-, 52-, and 46-kDa isoforms, respectively. For both PLCγ and Shc, EGF at 10 ng/ml induced a significantly greater increase in phosphorylation than IR (p < 0.05). Pretreatment of cells with 500 nm AG1478, a specific inhibitor of EGFR Tyr phosphorylation (29), abolished the radiation- and EGF-induced activation of both PLCγ and Shc in A431 cells. Levels of total PLCγ and Shc were not significantly altered by AG1478.

**Phosphorylation of EGFR Tyr-992 and Tyr-1173 by Radiation**

**(A) NON-RESPONSIVE**

- Y-1068: 2 Gy, IR = 6.3 ± 0.5, Tyr(P)
- Y-1068: 2 Gy, EGF = 4.4 ± 1.4, Tyr(P)
- Y-1086: 2 Gy, IR = 1.0 ± 0.9, Tyr(P)
- Y-1086: 2 Gy, EGF = 1.1 ± 0.1, Tyr(P)
- Y-1173: 2 Gy, IR = 1.0 ± 0.9, Tyr(P)
- Y-1173: 2 Gy, EGF = 1.1 ± 0.1, Tyr(P)

**Table 1**

| Phosphorylation site | Treatment | -fold change (mean ± S.E.) | Mean ratio ± S.E. (1 Gy/EGF) |
|---------------------|-----------|---------------------------|-----------------------------|
| Tyr(P) (total)      | 2 Gy⁴     | 3.1 ± 0.5                 | 0.5 ± 0.08                  |
| Tyr(P) (total)      | EGF       | 6.4 ± 1.4                 |                             |
| Tyr-1068            | 2 Gy      | 1.0 ± 0.9                 | 0.9 ± 0.04                  |
| Tyr-1068            | EGF       | 1.1 ± 0.1                 |                             |
| Tyr-1086            | 2 Gy      | 1.1 ± 0.2                 | 1.0 ± 0.1                   |
| Tyr-1086            | EGF       | 1.1 ± 0.08                |                             |
| Tyr-845             | 2 Gy      | 3.1 ± 1.1                 | 0.7 ± 0.1                   |
| Tyr-845             | EGF       | 5.3 ± 2.3                 |                             |
| Tyr-1045            | 2 Gy      | 3.3 ± 1.0                 | 0.5 ± 0.2                   |
| Tyr-1045            | EGF       | 9.5 ± 2.9                 |                             |
| Tyr-1173            | 2 Gy      | 2.1 ± 0.3                 | 0.6 ± 0.09                  |
| Tyr-1173            | EGF       | 3.6 ± 0.8                 |                             |
| Tyr-992             | 2 Gy      | 5.6 ± 1.9                 | 2.1 ± 0.3                   |
| Tyr-992             | EGF       | 2.8 ± 1.0                 |                             |

⁴ Mean of n = 4 independent experiments.

Expressions of either mutant abolished the radiation-induced increase in total Tyr phosphorylation, whereas the EGF-induced increase was only reduced by 50% (Fig. 3A). As expected, no significant signal relative to control levels was observed when the antibody against Tyr-992 (Fig. 3B) or Tyr-1173 (Fig. 3C) was used in combination with cells expressing the corresponding mutants, verifying the specificity of both mutant and antibody. Although CHO cells expressing EGFR-wt showed both radiation- and EGF-induced increases in Tyr-992 phosphorylation, the effect of radiation on Tyr-992 relative to Tyr-1173 was not as pronounced in CHO cells as in A431 cells. EGFR Dependence of Radiation-induced PLCγ and Shc Phosphorylation in A431 Cells...
Inhibition of Protein-tyrosine Phosphatases Enhances the Basal Tyr Phosphorylation of EGFR in Both A431 and EGFR-expressing CHO Cells with Tyr-992 Being the Most Significantly Affected—We demonstrated previously that radiation-induced EGFR phosphorylation is tightly regulated, peaking at 2 min following IR and recovering to basal levels over the next 10–30 min (Fig. 2). Phosphatases catalyze the dephosphorylation of Tyr-phosphorylated proteins either potentiating or antagonizing downstream cellular signaling. PTPs have been previously implicated in the dephosphorylation of EGFR (21, 23, 30, 31) with sequence specificity being evident for PTP1B and SHP2 at Tyr-992 (12, 13, 21, 22), and SHP-1 at Tyr-1173 (23).

One mechanism by which IR may promote sustained EGFR phosphorylation is by the disruption of dephosphorylation via inhibition of phosphatases. Previous studies demonstrated that therapeutic doses of IR result in the transient S-nitrosylation of the active site Cys of SHP1 and SHP2 with consequent inhibition of their catalytic activities (16). To test this hypothesis we used sodium vanadate to inhibit general phosphatase activity in both EGFR expressing CHO and A431 cells. CHO cells cultured in the presence of 100 μM sodium vanadate demonstrated enhanced and sustained EGFR Tyr phosphorylation. Total EGFR Tyr phosphorylation was increased 6-fold in sodium vanadate-treated CHO cells over that seen in the untreated control (Fig. 5A).

The vanadate-stimulated phosphorylation kinetics of Tyr-1068, Tyr-1173, and Tyr-992, one residue from each of the three categories described earlier were examined in CHO cells. The phosphorylation profiles of Tyr-1068 and Tyr-1173 matched that seen for total EGFR Tyr phosphorylation. Basal Tyr phosphorylation increased up to 10 min following introduction of sodium vanadate (Fig. 6A).

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tion of sodium vanadate and was sustained for the 30-min duration of the study (Fig. 5A). The magnitude of this increase in basal phosphorylation, however, was significantly greater for Tyr-992. In post-hoc pairwise comparisons of the areas under each curve (Turkey method to adjust for multiple testing), Tyr-992 phosphorylation was shown to be significantly more sensitive to inhibition of phosphatases by sodium vanadate than either Tyr-1068 or Tyr-1173 ($p = 0.0073$, and $0.01$ respectively). Total Tyr, Tyr-1173, and Tyr-1068 were not significantly different to each other ($p$ values ranging from 0.72 to 0.98).

Similarly in A431 cells Tyr-992 was significantly more sensitive to phosphatase inhibition than Tyr-1173, Tyr-1068, or total receptor Tyr (Fig. 5B, $p = 0.0013$, 0.0022, and 0.0008, respectively). In the presence of 100 μM sodium vanadate basal Tyr-992 phosphorylation was increased up to 8.6-fold, whereas both Tyr-1173 and total EGFR Tyr phosphorylation were only increased up to 3-fold. Tyr-1068 remained unchanged.
Inhibition of SHP2 Enhances Basal Phosphorylation of EGFR with Tyr-992 Being Significantly More Sensitive Than Total Tyr and Tyr-1173—SHP2 specifically has been shown to bind and dephosphorylate Tyr-992 in response to EGF (13), discriminating between EGFR and the other Tyr kinase receptors (31). Because IR in the therapeutic dose range has been shown to nitrosylate SHP2 with a consequent reduction in its phosphatase activity (16), we examined the role of SHP2 in regulation of EGFR phosphorylation. To do this we utilized a dominant negative SHP2 construct (c/s SHP2) mutated in its phosphatase active site.

CHO cells co-transfected with EGFR-wt and dominant negative SHP2 demonstrated enhanced phosphorylation of EGFR. Total Tyr phosphorylation was increased up to 2-fold over that seen in the control, whereas phosphorylation of Tyr-1173 was enhanced up to 2.5-fold (Fig. 6A). Phosphorylation of Tyr-992 was much more significantly affected, with an up to an 8-fold increase in phosphorylation over that of the mock-transfected control (p < 0.0001). In contrast overexpression of wt-SHP2 induced a 3-fold decrease in EGFR Tyr-992 phosphorylation. Phosphorylation of Tyr-1173 and EGFR were not significantly affected by overexpression of wt-SHP2. This data suggests that Tyr-992 is the most sensitive of the Tyr residues within EGFR to changes in phosphatase activity.

Similarly in A431 cells (Fig. 6B) Tyr-992 was significantly affected by knock-out of SHP2. Cells infected with a dominant negative SHP2 adenovirus exhibited an average 2.6-fold increase in Tyr-992 phosphorylation compared with a LacZ-infected control (p = 0.014). The phosphorylation of Tyr-1068 and -1173 remained unchanged. Unlike the CHO cells the wild type SHP2 expressing virus did not have a significant effect on the basal phosphorylation of Tyr-992. This may in part be explained by the endogenous expression of SHP2 in these cells. These cells may already express saturating levels of wt-SHP2 in which case they cannot respond further to the additional wt-SHP2 to which we are subjecting them. Previous studies also noted a lack of effect with the wt-SHP2 on global tyrosine phosphorylation of EGFR (12), reporting it to be because of the relatively few phospho-Tyr targets. The effect of the wt-SHP2 could therefore be masked by phosphorylation of Tyr residues unaffected by SHP2.

**DISCUSSION**

Earlier work established a fundamental role for EGFR in tumor cell resistance to IR via cytoprotective cell signaling (1). EGFR is phosphorylated in response to IR with consequent stimulation of signaling via the pro-proliferative and anti-apoptotic MAPK/PI3K and AKT pathways (1, 32, 33). Although several Tyr residues have been identified within the kinase domain of EGFR, to date no differentiation has been made between ligand-induced phosphorylation and that induced by IR. Because there have been various studies outlining the specificity of these Tyr residues for the different adaptor molecules and consequently downstream signaling from EGFR we quantified the phosphorylation of EGFR at the individual Tyr residues in response to EGF and IR.

In the present study we clearly defined three groups of Tyr residues within the kinase domain of EGFR. The first, which included Tyr-1068 and 1086, appeared completely unaffected by either radiation or EGF, possibly reflecting high constitutive phosphorylation levels. Previous work demonstrated Tyr-1068 to directly bind Grb2, an exchange factor involved in Ras stimulation, in response to EGF binding. A similar finding was reported with Tyr-1086 to be less responsive to EGFR. The second group, including Tyr-845, Tyr-1045, and Tyr-1173, although responsive to both EGF and radiation, exhibited a greater response to EGF than to radiation. Tyr-845 has been shown to be phosphorylated by Src, and this phosphorylation is involved in the regulation of EGF-stimulated DNA synthesis (26). Phosphorylation of Tyr-1045 creates a major docking site for c-Cbl, which binds to the activated EGFR (34). This leads to the assembly of the ubiquitination machinery on the receptor, en-
shown as mean -fold change in SHP2 association post-irradiation. EGFR was immunoprecipitated from cell lysates and prior to exposure to 2 Gy IR and lysed at 2, 5, 10, 30, and 60 min values were determined using an unpaired t test. EGFR was immunoprecipitated from cell lysates and subsequent Western blots probed for SHP2 and EGFR, respectively. Quantification data are shown as -fold change in SHP2 association as compared with an untreated control. Results are shown as mean ±fold change in SHP2 association ± S.E. (n = 3). p values were determined using an unpaired t test. B, A431 cells were starved for 16 h prior to exposure to 2 Gy IR and lysed at 2, 5, 10, 30, and 60 min post-irradiation. EGFR was immunoprecipitated from cell lysates and subsequent Western blots probed for SHP2. Quantification data are shown as mean -fold change in SHP2 association ± S.E. (n = 3). p values were determined using an unpaired t test.

abling receptor ubiquitination and consecutive degradation. Thus, exposure of cells to EGF and IR may also promote EGFR degradation as a negative feedback mechanism. Although Tyr-992 and Tyr-1045 were phosphorylated in response to both EGF and radiation they have not been directly associated with MAPK activation. Tyr-1173, however, has been shown to be a functional binding site for PLCγ and Shc (4, 5, 9–11), both of which could potentially mediate the activation of MAPK by EGF and IR (2).

The final group consists of one residue, Tyr-992, which was more highly phosphorylated in response to IR than EGF. Tyr-992 has also been shown to bind PLCγ and is believed to be the major site involved in activation of PLCγ (4, 5). This is consistent with the finding that mutation of residue Tyr-992 completely abolished radiation-induced EGFR phosphorylation, whereas it only blocked EGF-induced phosphorylation by 50%. These studies implicate Tyr-992 as an important effector in the radiation-induced activation of EGFR and downstream pathways. Tyr-1173 also contributes to the general induction of EGFR phosphorylation and mutation of Tyr-1173 also eliminated the radiation-induced increase in Tyr phosphorylation, indicating an important role for this molecule in radiation-induced signaling from EGFR. In A431 cells, however, this residue was more significantly affected by EGF than IR, suggesting that in the presence of the other ERBB molecules it is not as IR-specific as Tyr-992. We have shown previously that EGFR tumor cells exists in both homo- and heterodimers with itself and ERBB2 or ERBB4 (35). Therefore the magnitude of EGFR phosphorylation in response to IR may well rely on heterodimerization with ERBB2 and/or ERBB4.

Another interesting observation is the increase in basal phosphorylation of EGFR when Tyr-992 or -1173 are mutated. This is not surprising when one considers the specificity of these two sites for binding of PTPs. Studies of RTKs, including EGFR, demonstrate that the overall phosphorylation state in unstimulated cells is a net result of basal RTK and PTP activities (35, 36). Because the catalytic activity of PTPs can be up to 1000-fold greater than that of kinases (37), perturbation of PTP activity may have a significantly more profound effect on signal propagation than that of kinases. It has also been shown, at least in response to ROS, that activation of EGFR is coupled to the inhibition of PTP via hydrogen peroxide production with a consequent amplification of ligand-induced activation (17–20).

In accordance with the minimal network strategy described by Reynolds et al. (37) inhibition of endogenous PTP activity by sodium vanadate increased the basal phosphorylation of EGFR in both EGFR expressing CHO and A431 cells. In cells overexpressing EGFR, constitutive basal phosphorylation of EGFR is commonly observed, and is reported to be because of autocrine stimulation by ligands such as transforming growth factor-α. The consequent survival signaling supports a cellular growth advantage in EGFR overexpressing tumor cells. We have clearly demonstrated in this study that this phosphorylation is particularly sensitive to changes in phosphatase activity.

The unique finding in our study was the much more dramatic effect that PTP inhibition had on Tyr-992 compared with the other residues. This was evident in both EGFR expressing CHO cells and A431 cells in which this residue is critical for IR-induced phosphorylation. Consequently we focused our attention on the Tyr-992-specific PTP, SHP2, and found inhibition of endogenous SHP2 activity to enhance EGFR phosphorylation. Again Tyr-992 was significantly more sensitive than either Tyr-1173 or total receptor Tyr phosphorylation. This data suggests that not only is Tyr-992 specifically involved in IR-induced signaling from EGFR but it is uniquely modulated by PTPs, particularly SHP2. Although we corroborated the finding that Tyr-992 is essential for SHP2 action (13), previous data all pertains to ligand-induced EGFR stimulation. In our study we demonstrated a unique association between SHP2 and EGFR and this association is maintained for up to 20 min following dephosphorylation of the receptor. This may reflect a dual role for SHP2 as both a phosphatase and an adapter molecule. In addition to a phosphatase domain, responsible for recognition and dephosphorylation of the phosphorylated Tyr residue, SHP2 also has both C- and N-terminal SH2 domains (39, 40). Both of these sites face away from the active phosphatase domain and are potentially available to interact with other
Phosphorylation of EGFR Tyr-992 and Tyr-1173 by Radiation

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