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Physical Interaction between Epidermal Growth Factor Receptor and DNA-dependent Protein Kinase in Mammalian Cells*

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Binding of extracellular ligands to epidermal growth factor receptors (EGFR) activate signal transduction pathways associated with cell proliferation, and these events are inhibited by monoclonal antibodies against EGFR. Since efficient DNA repair in actively growing cells may require growth factor signaling, it was of interest to explore any linkage between EGFR-mediated signaling and DNA-dependent protein kinase (DNA-PK), an enzyme believed to be involved in repairing double strand breaks and V(D)J recombination. We report that anti-EGFR monoclonal antibodies (mAbs), and not EGFR ligands, trigger a specific early physical interaction between EGFR and a 350-kDa catalytic subunit of DNA or its regulatory heterodimeric complex Ku70/80, in a variety of cell types, both *in vivo* and *in vitro*. Inhibition of EGFR signaling by anti-EGFR mAb was accompanied by a reduction in the levels of the DNA-PK and its activity in the nuclear fraction. Confocal imaging revealed that a substantial amount of DNA-PK was co-localized with EGFR in anti-EGFR mAb-treated cells. Anti-EGFR mAb-induced physical interaction between EGFR and DNA-PK or Ku70/80 was dependent on the presence of EGFR, but not on the levels of EGFR. The EGFR associated with DNA-PK or Ku70/80 retains its intrinsic kinase activity. Our findings demonstrate the existence of a novel cellular pathway in mammalian cells that involves physical interactions between EGFR and DNA-PK or Ku70/80 in response to inhibition of EGFR signaling. Our present observations suggest a possible role of EGFR signaling in maintenance of the nuclear levels of DNA-PK, and interference in EGFR signaling may possibly result in the impairment of DNA repair activity in the nuclei in anti-EGFR mAb-treated cells.

The mitogenic effects of growth stimulatory polypeptides are regulated by their binding to high affinity transmembrane receptors with intrinsic kinase activity (1–3). The epidermal growth factor is one of the well characterized growth factor that stimulates the proliferation of fibroblasts and most types of epithelial cells (4). Modifications of EGFR[1] induced by extracellular ligands such as EGF or TGF-α lead to engagement of EGFR with signal transduction pathways associated with mitogenesis and cell proliferation (1–5). Overexpression of EGFR, as well as its natural ligand TGF-α, has been shown to induce transformed properties in recipient cells (6). In recent years, approaches involving interference with and/or blocking of EGFR-mediated growth stimulation by anti-EGFR monoclonal antibodies have been the subject of active investigation in an effort to control cell proliferation (7). mAbs 528 and 225, raised against the extracellular domain of EGFR, have been shown to bind to the receptors with high affinity comparable with the natural ligand, compete for binding of TGF-α or EGF, and block both ligand-induced activation of EGFR kinase activity and growth stimulation of normal and malignant cells (8–13). The anti-EGFR mAb-mediated inhibition of cell proliferation has been attributed, at least in part, to the EGFR dimerization (13). Other cellular effects of blockade of the EGFR signaling by anti-EGFR mAbs include inhibition of cell cycle progression (14), induction of apoptosis (15),[2] enhanced chemosensitization (17, 18), and inhibition of the repair efficiency of the damaged DNA (reviewed in Ref. 19). Anti-EGFR mAbs have been shown to block the capacity of ligand to activate receptor tyrosine kinase in intact cells (13), but the kinase activity can be detected in EGFR precipitated from cell lysates with anti-receptor mAbs (8, 12).

The DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that consists of a 350-kDa catalytic subunit and a heterodimeric regulatory complex Ku70/80 and can *in vitro* phosphorylate a number of transcription factors and DNA binding proteins, including p53, the RNA polymerase II carboxyl-terminal domain, replication factor A, and the Ku (20–23). The carboxyl-terminal 380 amino acids of DNA-PK exhibits sequence homology with the catalytic domains of members of the phosphatidylinositol (PI) 3-kinase superfamily (23). The activity of DNA-PK has been shown to be inhibited by autophosphorylation (24). The DNA-PK is believed to play a major role in repairing double strand DNA breaks and V(D)J recombination, as mutations of DNA-PKcs cause both x-ray sensitivity and defective V(D)J recombination (17, 23). Since efficient DNA repair may require growth factor signaling (19, 25), it is of interest to explore any linkage between EGFR-mediated signaling and DNA-PK pathway. We report the results of experiments demonstrating specific early physical interaction between EGFR and DNA-PK or Ku70/80 in mammalian cells in

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; mAb, monoclonal antibodies; DNA-PK, catalytic subunit of DNA-dependent protein kinase; TGF, transforming growth factor; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate; immunoprecipitation.

[2] M. Mandal, L. Adam, J. Mendelsohn, and R. Kumar, submitted for publication.

response to anti-EGFR mAbs and not in response to EGFR ligands.

MATERIALS AND METHODS

Cell Culture and Immunoblotting—DiFi human colorectal carcinoma cells (15, 26, 27),[2] HER14 murine fibroblasts expressing human EGFR (13, 28), A431 human epidermoid carcinoma cells (9), and MDA-MB453 human breast carcinoma cells (29) were maintained in Dulbecco's modified Eagle's medium-F-12 (1:1; Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.). HeLa human cervical carcinoma cells (30) and U266 leukemic cells (ATCC) were cultured in minimal essential medium (Life Technologies, Inc.) and RPMI (Life Technologies, Inc.), respectively, supplemented with 10% fetal calf serum. Antibodies against DNA-PK (clones 24-5, 18-2 and 42-psc), Ku70/80, and EGFR (clones 11E8 and H11) were purchased from the Neomarker Inc. (Fremont, CA).

For preparation of cell extracts, cells were were washed three times with phosphate-buffered saline and lysed in buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.4% Nonidet P-40, 100 mM NaF, 200 μM $NaVO_5$, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml apotinin) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. Cell lysates containing equal amounts of protein were resolved on a 7% SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies by using an ECL method or alkaline phosphatase-based color reaction method (30, 31).

Metabolic Labeling and Immunoprecipitation—An equal number of cells were metabolically labeled for 6 or 3 h with 100 μCi/ml [^{35}S]methionine in methionine-free medium containing 2% fetal bovine serum in the absence or presence of mAb 225. Alternately, cells were labeled for 3 h and treated with mAb for the last 30 min of culture before harvesting. Cells extracts were prepared as described above. Cell extracts containing an equal amount of total trichloroacetic acid-perceptible counts were immunoprecipitated with the desired mAb or control rabbit anti-mouse-protein A conjugate, resolved on a SDS-polyacrylamide gel, and analyzed by autoradiography (29–32).

EGFR Intrinsic Kinase Assay—Cell lysates were immunoprecipitated with EGFR or DNA-PK or Ku70/80 mAbs, and EGFR kinase activity was measured by a immune complex kinase assay as described (33). Reaction products were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by autoradiography. When indicated, subsequent to autoradiography, the blot was immunoblotted with the appropriate mAbs to confirm the identity of phosphorylated protein bands.

Subcellular Fractionation—Cytosolic and nuclear extracts were prepared from unlabeled or metabolically labeled cells as described (32, 34). Briefly, cells were washed with phosphate-buffered saline and pelleted at 1500 rpm for 5 min. Cells were resuspended in 200 μl of ice-cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride) and by gently pipetting up and down 10 times and left on ice for 5 min. The lysate was spun for 1 min to separate nuclei, and supernatant was transferred to a new tube. For cytosol preparation, the supernatant was centrifuged at 15,000 rpm for 15 min. Nuclei were washed with 500 μl of lysis buffer and resuspended in 200 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride), vigorously shaken at 4 °C for 15 min, centrifuged at 15,000 rpm for 15 min, and the supernatant (nuclear extracts) was transferred to a new tube.

DNA-PK Activity Assay—For assaying the DNA-PK kinase activity, cytosolic and nuclear fractions (35) were prepared and assayed using a peptide substrate (EPPLSQEAFADLWKK, purchased from the Promega) specific for DNA-PK as per the manufacture's instructions (36, 37). As control, DNA-PK kinase reaction was performed with or without enzyme or DNA or peptide substrate. Purified DNA-PK (Promega) was used as a positive control.

Immunofluorescence Confocal Studies—Cellular localization of DNA-PK was determined using indirect immunofluorescence as described (38), with some modification. Briefly, cells grown on glass coverslips were fixed (without permeabilization) in acetone at −20 °C for 3 min. Several dilutions of each antibody were used to obtain the optimal results. For staining of DNA-PK, DiFi cells were treated with or without anti-DNA-PK mAb 25–4 followed by TRITC-labeled goat anti-mouse secondary antibody (Molecular Probes). For controls, cells were treated only with the TRITC-goat anti-mouse antibody, omitting the primary antibody, and no signals were detected in control untreated cells. Also, there was no significant staining background by either FITC-goat antibody or by rabbit EGFR antibody. Each image represents Z sections at the same cellular level and magnification. Confocal analysis was performed using a Zeiss laser scanning confocal microscope using the established methods, involving processing of the same section for each detector (the two excitations corresponding to FITC and TRITC) and comparing pixel by pixel. Co-localization of two proteins is demonstrated by the development of yellow color due to red and green pixels.

RESULTS AND DISCUSSION

During an investigation on the expression of DNA-PK in DiFi human colorectal cells (15)[2] treated with anti-EGFR mAb 225, we observed that immunoprecipitation (IP) of ^{35}S-labeled cell lysates with an antibody (clone 25-4) against the catalytic subunit of DNA-PK (DNA-PK) co-immunoprecipitated a protein of approximate molecular mass of 170 kDa in mAb 225-treated cells, but not in control cells (Fig. 1*A*). Based on the size of 170 kDa, we explored the possibility that the observed DNA-PK-associated protein could be EGFR. Supernatants from DNA-PK IP were re-immunoprecipitated with anti-EGFR mAb 528 (9, 10). Results indicated that there was almost complete absence of EGFR in lysates of mAb 225-treated cells that had been subjected to IP with anti-DNA-PK mAb (Fig. 1*B*). The *lower panel* in Fig. 1*B* shows the levels of total EGFR protein in mAb 225-treated lysates of mAb 225-treated DiFi cells as determined by direct immunoblotting with an anti-EGFR mAb (clone 11E8, raised against a fusion protein containing 330 amino acids of the cytoplasmic domain of EGFR) (39). Since activation of DNA-PK requires interaction with its regulatory subunits Ku70 and Ku80, ^{35}S-labeled cell lysates from control and mAb 225-treated (3 h) DiFi cells were immunoprecipitated with an anti-Ku70/80 mAb that recognizes the Ku70/80 heterodimer or with anti-EGFR mAb 528. Treatment of DiFi cells with mAb 225 also resulted in increased association of a 170-kDa protein band with Ku70/80 (Fig. 1*C, lanes 5* and *6*). As expected from previous studies (9, 10), mAb 225 treatment resulted in a modest reduction in newly synthesized EGFR (Fig. 1*C, lanes 3* and *4*). However, re-IP of supernatants from *lanes 3* and *4* with anti-DNA-PK mAb 25-4 demonstrated a significant increase in the levels of co-IP 170-kDa protein band with DNA-PKcs only in mAb 225-treated cell lysates (Fig. 1*C, lanes 7* and *8*). Co-immunoprecipitation of ^{35}S-labeled 170-kDa protein with DNA-PK was also observed with two other anti-DNA-PK mAbs, clones 18-2 and 42-psc (data not shown). Results in Fig. 1*D* demonstrate the specific interaction between DNA-PK and EGFR in DiFi cells treated with mAb 225 for 30 min. In brief, these observations indicated that treatment of DiFi cells with anti-EGFR mAb 225 triggers early physical interaction of a 170-kDa protein (presumably EGFR) with both catalytic and regulatory subunits of DNA-PK.

To examine whether the observed interaction between 170-kDa protein and DNA-PK could be also detected during EGFR activation by its ligand, we compared the effect of TGF-α treatment (30 min) on metabolically labeled DiFi cells with that of anti-EGFR mAbs 225 and 528-treated (30 min) cells. As a control, an unrelated antibody 4D5 against HER2 receptor (29) was used. DiFi cells have easily detectable levels of HER2 (Fig. 2*B*). As expected, activation of EGFR by its ligand TGF-α, but not anti-EGFR mAbs, resulted in a mobility shift of EGFR due to increased phosphorylation (Fig. 2*A, upper panel, lanes 2* and *3*). In contrast, in spite of EGFR activation by TGF-α (or EGF, data not shown), there was no co-IP of a 170-kDa protein band with DNA-PK. Treatment of DiFi cells with both anti-EGFR mAbs 225 and 528, and not 4D5, resulted in a rapid association of 170-kDa protein with DNA-PKcs (Fig. 2*A, middle panel, lanes 4* and *5*). Association of the 170-kDa EGFR protein band with anti-DNA-PK immunoprecipitates from mAb 225-treated DiFi cells was readily detectable by staining with Coomassie Brilliant Blue (Fig. 2*A, lower panel*). To further establish the

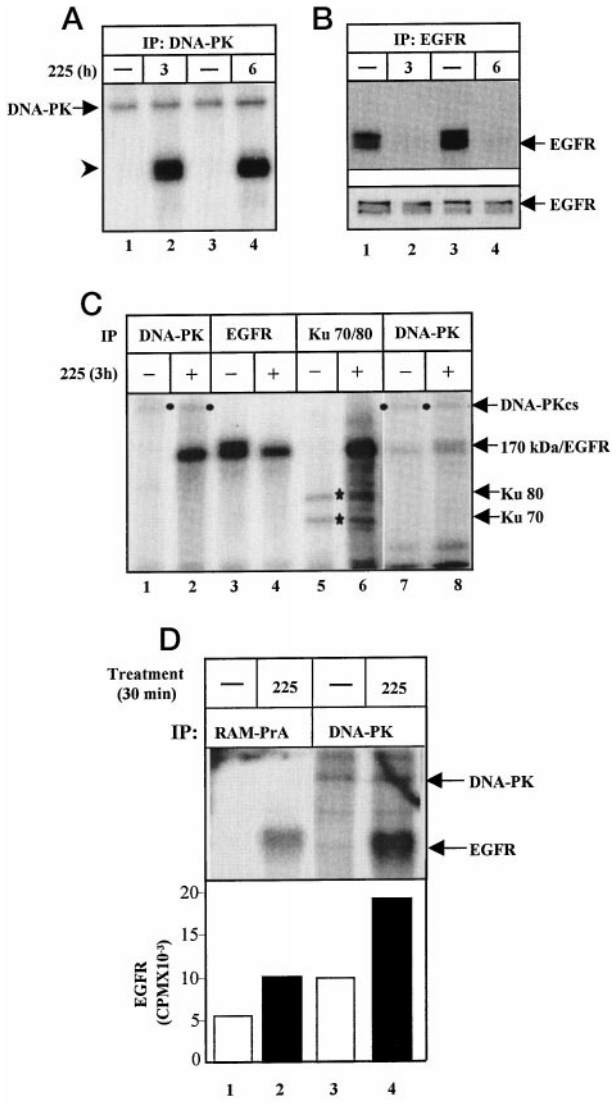

FIG. 1. **Anti-EGFR mAb 225 induces association of a 170-kDa protein with DNA-PK or Ku70/80.** *A*, DiFi cells were metabolically labeled with 100 μCi/ml [^{35}S]methionine for 3 h (*lanes 1* and *2*) or 6 h (*lanes 3* and *4*) in the presence or absence of 30 nM mAb 225. Total cells extracts containing an equal amount of total trichloroacetic acid-precipitable counts (or equal amount of protein in case of unlabeled cell lysates) were immunoprecipitated with anti-DNA-PK mAb 25-4 and analyzed by SDS-PAGE and autoradiography. *B*, supernatants from the above DNA-PK IP were re-immunoprecipitated with anti-EGFR mAb 528 and analyzed as above. *Lower panel*, cell lysates were immunoblotted with anti-EGFR mAb 11E8 using alkaline phosphatase color method. *C*, DiFi cells were labeled with [^{35}S]methionine for 3 h in the presence or absence of 30 nM mAb 225. Total cell lysates were immunoprecipitated with anti-DNA-PK mAb 25-4 (*lanes 1* and *2*) or anti-EGFR mAb 528 (*lanes 3* and *4*) or anti-Ku70/80 mAb (*lanes 5* and *6*). Supernatants from *lanes 3* and *4* were re-immunoprecipitated with anti-DNA-PK mAb 25-4 (*lanes 7* and *8*) and analyzed by SDS-PAGE and autoradiography. ●, DNA-PK; *, Ku70/80. *D*, DiFi cells were labeled with [^{35}S]methionine for 3 h and treated with or without 30 nM mAb 225 during the last 30 min before harvesting. Cell lysates containing equal amount of trichloroacetic acid-perceptible counts were immunoprecipitated with anti-DNA-PK mAb 25-4 (*lanes 3* and *4*) or control rabbit anti-mouse (*RAM*)-protein A conjugate (*lanes 1* and *2*). Samples were resolved on a 7% SDS-PAGE and analyzed by autoradiography. Quantitation of radioactivity associated with the EGFR band is shown in the *lower panel*.

identity of 170-kDa protein as EGFR, we next immunprecipitated cell lysates from DiFi cells treated with TGF-α or mAb 225 for 30 min and immunoblotted with anti-DNA-PK mAb or anti-EGFR mAb 11E8 or Ku70/80 mAb. Results in Fig. 2*C* confirmed that mAb 225 induces the association between

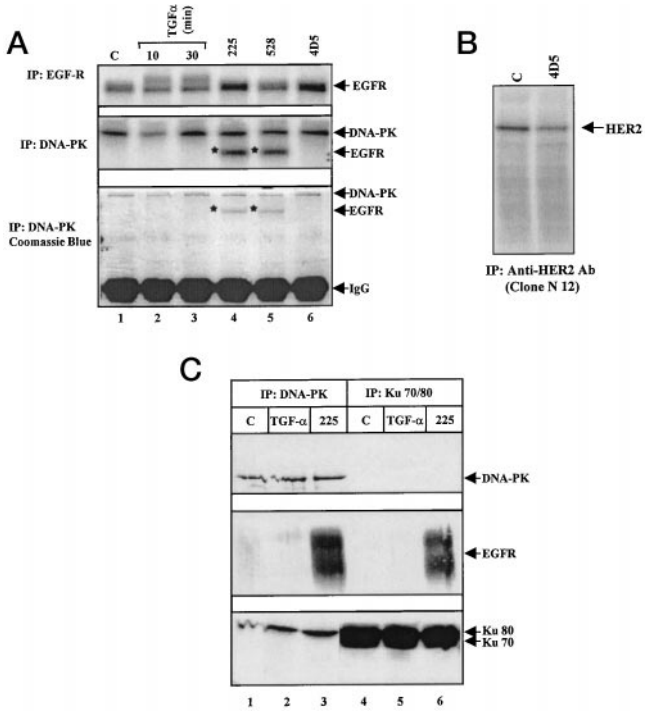

FIG. 2. **Anti-EGFR mAbs, and not EGFR ligands, trigger interaction between EGFR and DNA-PK or Ku70/80.** *A*, DiFi cells were metabolically labeled with [^{35}S]methionine for 3 h and treated with or without 30 nM TGF-α (*lanes 2* and *3*) or 30 nM mAb 225 (*lane 4*) or 30 nM mAb 528 (*lane 5*) or 30 nM mAb 4D5 (*lane 6*) for 30 min before cells harvesting. Cell lysates were immunoprecipitated with anti-EGFR mAb 528 and analyzed as SDS-PAGE and autoradiography (*upper panel*). Cell lysates from the above samples were immunoprecipitated with anti-DNA mAb 25-4 and analyzed by autoradiography (*middle panel*) or Commassie Brilliant Blue (*lower panel*). *B*, ^{35}S-labeled DiFi cells were treated with or without 30 nM mAb 4D5 for 30 min. Cell lysates were immunoprecipitated with another anti-HER2 mAb clone N12 (Neomarker). *C*, DiFi cells were treated for 30 min with or without 30 nM TGF-α (*lanes 2* and *5*) or 30 nM mAb 225 (*lanes 3* and *6*). Cell lysates were immunoprecipitated with anti-DNA-PK mAb 25-4 (*lanes 1–3*) or anti-Ku70/80 mAb (*lanes 4–6*) and blotted with anti-DNA-PK mAb (*upper panel*) or anti-EGFR mAb 11E8 (*middle panel*) or Ku70/80 mAb (*lower panel*) from the same blot.

EGFR and DNA-PK (*lane 3*) or Ku70/80 (*lane 6*). The association of EGFR with DNA-PK was also established by stripping the above blots and re-immunoblotting with an another anti-EGFR polyclonal antibody RK2 (Ref. 40, raised against a peptide corresponding to amino acids 985–996 of EGFR) or with a different anti-EGFR-mAb H11 (Ref. 41, raised against extracellular domain of EGFR) (data not shown).

mAb-induced interaction between EGFR and DNA-PK or Ku70/80 in mAb 225-treated cells was specific to mAb 225, which also induced physical interaction between EGFR and DNA-PK or Ku70/80 in murine fibroblast NIH 3T3 cells engineered to express human EGFR (HER14 cells; Ref. 16) (Fig. 3*A*, *lanes 2* and *5*). Data in Fig. 3*B* demonstrate that the observed interaction between EGFR and DNA-PK in mAb 225-treated HER14 cells could be also detected by immunoprecipitation of cell lysates with anti-EGFR mAb.

To determine whether DNA-PK-associated EGFR was functionally active, we next performed a EGFR *in vitro* intrinsic kinase assay using anti-DNA-PK and anti-Ku immunoprecipitates. As a positive control, anti-EGFR immunoprecipitates from control and TGF-α-stimulated A431 cell lysates were used (Fig. 4*A*, *lanes 1* and *2*). The EGFR tyrosine kinase displayed the capacity to autophosphorylate EGFR in both anti-DNA-PK and anti-Ku immunoprecipitates from mAb 225-treated DiFi cells (Fig. 4*A*). There was no effect of TGF-α or mAb 225

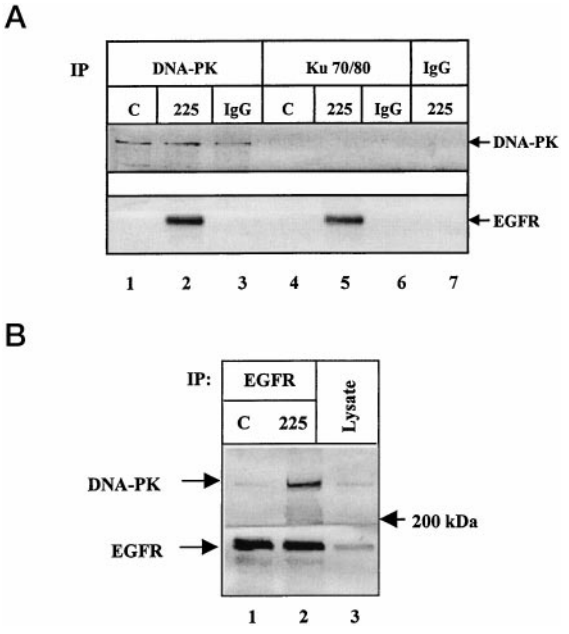

FIG. 3. *A*, HER14 cells were treated with or without 30 nM mAb 225 or control IgG for 30 min. Cell lysates were immunoprecipitated with DNA-PK (*lanes 1–3*) or Ku70/80 (*lanes 4–6*) or control mouse IgG (*lane 7*) and blotted with DNA-PK (*upper panel*) or EGFR (*lower panel*) mAbs from the same blot. *B*, HER14 cells were treated with or without 30 nM mAb 225 for 30 min, and cell lysates were immunoprecipitated with anti-EGFR mAb 528 and blotted with DNA-PK mAb (*upper panel*) or anti-EGFR mAb 11E8 (*lower panel*) from the same blot.

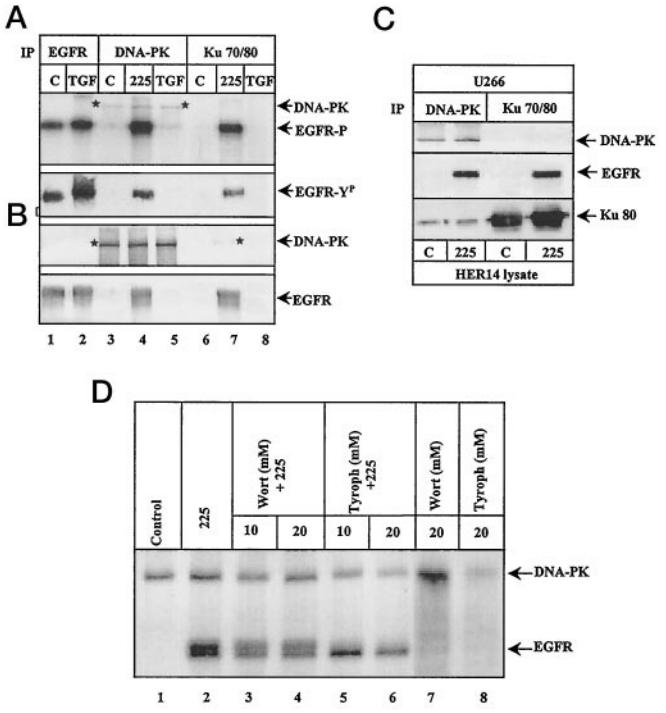

FIG. 4. **Autophosphorylation of EGFR associated with DNA-PK.** *A*, DiFi cells were treated DiFi cells were treated for 30 min without or with 30 nM TGF-α (*lanes 5* and *8*) or 30 nM mAb 225 (*lanes 4* and *7*). Two sets of cell lysates were immunoprecipitated with anti-DNA-PK mAb (*lanes 3–5*) or anti-Ku70/80 mAb (*lanes 6–8*). As EGFR kinase control, A431 cells were treated with 30 nM TGF-α for 15 min and immunoprecipitated with anti-EGFR mAb 528 (*lanes 1* and *2*). After immunoprecipitated, one set of precipitated EGFR was subjected to *in vitro* kinase assay, analyzed by SDS-PAGE, transferred to nitrocellulose, and autoradiograph resulted from 1-h exposure is shown here (*upper panel*). The second set of precipitated EGFR was analyzed by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphate-Tyr. mAb PY-20 (*lower panel*). *B*, to confirm the identity of phosphorylated protein bands in *A* (*upper panel*), subsequent to autoradiography, the membrane was blotted with DNA-PK or EGFR mAbs, using a color reaction method of detection (as in Fig. 1*B, lower panel*). *C*, DNA-PK associates with EGFR *in vitro*. DNA-PK and Ku70/80 were purified from U266 cells by IP, and the beads were further incubated for 2 h with cell lysates from control and mAb 225-treated (30 nM) HER14 cells. Beads were analyzed by SDS-PAGE and immunoblotted with DNA-PK (*upper panel*) or EGFR (*middle panel*) or Ku80 (*lower panel*) mAbs from the same blot. *D*, DiFi cells were labeled with [^{35}S]methionine for 3 h and treated with or without 30 nM mAb 225 in the presence or absence of wortmannin (*Wort*) (Sigma) or tyrphostin A9 (*Tyroph*) (Calbiochem) for 30 min before preparing cell lysates. Cell lysates were immunoprecipitated with anti-DNA-PK mAb 25–4 and analyzed by SDS-PAGE and autoradiography.

treatment upon the status of DNA-PK phosphorylation in these assays (Fig. 4*A, lanes 3–5*). Subsequent to autoradiography, the identity of the phosphorylated protein bands was confirmed by immunoblotting with specific mAbs against DNA-PK or EGFR (Fig. 4*B*). There was no effect of mAb 225 treatment on the activity of DNA-PK in cell lysates, using GAL4-CTD (34, 35) as a substrate (data not shown). Taken together, these results demonstrated that treatment of DiFi cells with anti-EGFR mAb 225, but not with EGFR ligands, leads to interaction of EGFR with DNA-PKcs or Ku70/80.

To further study the interaction between EGFR and DNA-PK, we next performed mixing experiments involving isolation of DNA-PK or Ku70/80 from U266 human leukemic cells that lack EGFR and then incubation with cell lysates from HER14 cells. DNA-PK and Ku70/80 immunoprecipitates from U266 cells were incubated for 2 h with cell lysates from control or mAb 225-treated HER14 cells, and the beads were washed and immunoblotted with anti-EGFR mAb 11E8 or control antibodies. Results in Fig. 4*C* demonstrate that EGFR from mAb 225-treated HER14 cells, and not from untreated HER14 cells, interacted with DNA-PK and Ku70/80. DNA-PK has been shown to have significant homology with PI 3-kinase, which is a downstream target of EGFR signaling. Most, if not all, known functions of EGFR require EGFR kinase activity. We examined the involvement of PI 3-kinase and EGFR kinase in mediating the interaction between DNA-PK and EGFR. The PI 3-kinase inhibitor wortmannin and the EGFR kinase inhibitor tyrphostin A9 did not prevent the interaction between EGFR and DNA-PK in DiFi cells (Fig. 4*D*). As expected, tyrphostin A9 reduced the phosphorylation of EGFR, as demonstrated by the band shift in *lanes 5* and *6*.

DNA-PK has been shown to be primarily localized in the nucleus (21–23). Our finding of physical interaction between EGFR and DNA-PK has raised the possibility of redistribution of DNA-PK in response to anti-EGFR mAbs. To examine the effect of mAb 225 on the subcellular localization of DNA-PK, DiFi cells were treated with mAb 225 for 30 min, and cytosolic and nuclear extracts were prepared. As expected, a major fraction of DNA-PK was localized in the nucleus and a minor fraction in the cytosol (Fig. 5*A, upper panel*), and EGFR was present in the cytosol (Fig. 5*A, middle panel*). Treatment with mAb 225 was accompanied by a reduction in the level of nuclear DNA-PK. Ku80 was localized in the cytosolic and nuclear extracts. Subcellular fractionation of ^{35}S-labeled DiFi cells treated with mAb 225 indicated that EGFR interacts with the cytosolic fraction of DNA-PK (Fig. 5*B, lane 2*) and also confirmed a decrease in the amount of the nuclear DNA-PK (Fig. 5*B*, compare *lane 4* with *lane 3*). To determine whether the observed reduction in the amount of the nuclear DNA-PK is functional, we measured the DNA-PK kinase activity in the cytosolic and nuclear fractions from control and mAb 225-treated DiFi cells using a peptide substrate specific for DNA-PK (36, 37). Results in Fig. 5*C* demonstrate that DNA-PK activity was primarily localized in the nuclear fraction in con-

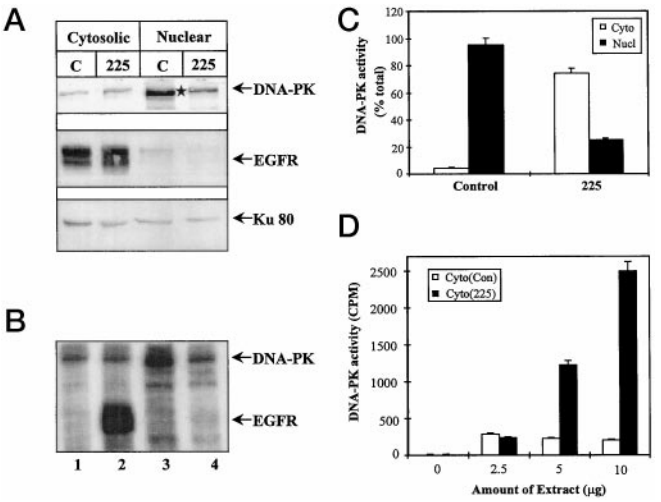

FIG. 5. **Effect of mAb 225 on subcellular distribution of DNA-PK.** *A*, cytosolic and nuclear extracts from control and mAb 225-treated DiFi cells were immunoblotted with DNA-PK (*upper panel*) or EGFR (*middle panel*) or Ku80 (*lower panel*) mAbs. *B*, DiFi cells were labeled with [^{35}S]methionine for 3 h and treated with (*lanes 2* and *4*) or without (*lanes 1* and *3*) 30 nM mAb 225 for 30 min. Cytosolic and nuclear extracts were immunoprecipitated with anti-DNA-PK mAb 25-4 and analyzed by SDS-PAGE and autoradiography. *C*, 10 μg of cytosolic (*open bars*) or nuclear (*solid bars*) extracts from control or mAb 225-treated DiFi cells were assayed for DNA-PK activity as described (34). Results are expressed as the percentage of total activity (100%) in each fraction. *D*, increasing amount of cytosolic extracts from control (*open bars*) or mAb 225-treated (*solid bars*) DiFi cells were assayed for DNA-PK activity. Results are expressed as the total count per minute (*CPM*) incorporated in the peptide substrate.

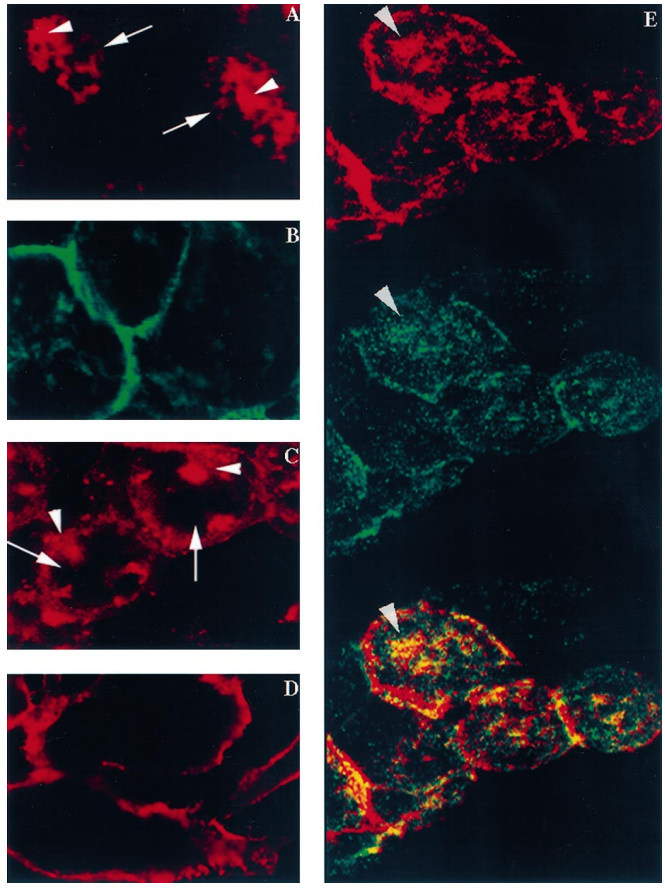

FIG. 6. **Immunofluorescence co-localization of EGFR and DNA-PK in DiFi cells.** Cells were grown on coverslip and treated with mAb 225 (*C, D*, and *E*) or without mAb (*A* and *B*) for 30 min and fixed. After blocking, cells were double-stained using affinity-purified mouse anti-DNA-PK mAb (*A, C*, and *E, upper* and *bottom panels*), and rabbit anti-EGFR Ab (*B* and *E, middle* and *bottom panels*). Control pattern of staining due to mAb 225 is shown in *D*. Primary antibodies (DNA-PK and mAb 225) were detected using rhodamine (TRITC)-conjugated goat-anti-mouse IgG or fluorescein-conjugated goat-anti-rabbit IgG for the detection of EGFR. *Large arrowheads* in *A* and *C* show nuclear or extranuclear localization of DNA-PK (*red staining*). *Yellow color* in *E* (*bottom frame*) represents co-localization of *red* and *green colors*; extranuclear co-localization of internalized EGFR and DNA-PK is shown by the *arrowhead*; yellow color on the outer membrane represents co-localization of surface membrane-associated EGFR and mAb 225.

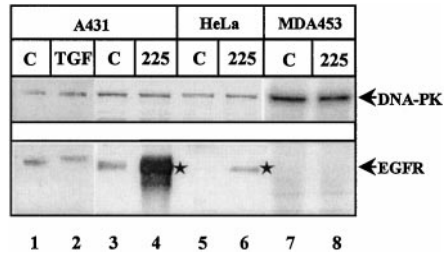

FIG. 7. **Interaction between EGFR and DNA-PK or Ku70/80 in diverse cell types.** A431 cells, HeLa cells, and MDA-MB-453 cells were treated without or with 30 nM mAb 225 (*lanes 4, 6*, and *8*) or TGF-α (*lane 2*) for 30 min. Cell lysates were immunoprecipitated with DNA-PK and blotted with DNA-PK (*upper panel*) or EGFR (*lower panel*) mAbs.

trol cells, and treatment with mAb 225 was accompanied with 75% reduction in the levels of the nuclear DNA-PK activity. Data in Fig. 5*D* illustrate the effect of mAb 225 on the activity of DNA-PK in the cytosolic fraction as a function of enzyme concentration.

The observed interaction between EGFR and DNA-PK in mAb 225-treated cells was further validated by confocal microscopy (Fig. 6). mAb 225 treatment of DiFi cells was accompanied by a significant reduction in the amount of DNA-PK (shown by *arrowheads*) in the nucleus (shown by *arrows* that are located just under the nuclear membrane) (*panel C*). As a control, mAb 225-treated DiFi cells were stained without DNA-PK mAb (Fig. 6*D, red staining*). Results in Fig. 6*E* demonstrate the development of *yellow color* due to co-localization of *red* (DNA-PK) and *green* (internalized EGFR) pixels in mAb 225-treated cells (shown by *arrowhead*). In brief, these results revealed that a substantial amount of DNA-PK was co-localized with the internalized EGFR in anti-EGFR mAb-treated cells.

To examine the generality of physical interaction between EGFR and DNA-PK, we extended our investigation to A431 human epidermoid carcinoma cells (which express high level of EGFR), HeLa human cervical carcinoma cells (which express normal level of EGFR), and MDA453 human breast carcinoma cells (which lack EGFR). mAb 225 treatment also stimulated the interaction between EGFR and DNA-PK in A431 cells and HeLa cells (Fig. 7, *lanes 4* and *6*), but not in EGFR minus MDA453 cells (*lanes 7* and *8*). Data in Fig. 7 also demonstrate that there was some base-line interaction between EGFR and DNA-PK in A431 cells (*lanes 1* and *3*), which was specifically increased by mAb 225 (*lane 4*), but not by TGF-α (*lane 2*). In brief, our results suggested that the observed interaction between EGFR and DNA-PK in mAb 225-treated cells was dependent on the presence of EGFR, but not the levels of EGFR.

mAbs against the extracellular domain of EGFR have been widely considered as potential antiproliferatve therapeutic agents. Our findings demonstrate that incubating cells with anti-EGFR mAbs, and not with EGFR ligands, triggers a specific physical interaction between EGFR and DNA-PKcs and EGFR and Ku70/80 in a variety of cell types. This can be demonstrated both *in vivo* and *in vitro*. The anti-EGFR mAb-induced interaction between EGFR and DNA-PK or Ku70/80 was dependent on the presence of EGFR, but not on the amount

of receptor. The tyrosine kinase in DNA-PK-associated EGFR remains active in the anti-EGFR mAb-treated cells. The potential significance of the observed interactions between EGFR and DNA-PK and Ku70/80 in the response to anti-EGFR mAbs remains to be delineated. Some of the known functions of DNA-PK include repairing double strand breaks and V(D)J recombination and phosphorylation of a number of transcription factors (21–23). In this context, our finding that mAb 225 induces a reduction in the level of DNA-PK in the nucleus is important, as it implies a possible role of EGFR-signaling in maintenance of the nuclear levels of DNA-PK, and interference in EGFR signaling may possibly result in the impairment of DNA repair activity in the nuclei of anti-EGFR mAb-treated cells.

Our findings raise new questions about the intracellular events that results from blockade of EGFR tyrosine kinase by anti-EGFR mAbs. Inhibition of signal transduction pathways activated by the EGFR tyrosine kinase may alter the status of proteins that mediate interaction between EGFR and DNA-PK, and the reduced phosphorylation status of the receptor may facilitate the interaction. Alternatively, receptor dimerization (without kinase activation) induced by mAb 225 may activate a signal that leads to the observed interaction, since kinase inactivation by tyrphostin A9 in the absence of mAb 225 did not result in EGFR-DNA-PK interaction. The roles of the EGFR and the DNA-PK associated with each other, and the structure-function relationship between EGFR and DNA-PK requires further experimentation. In summary, our findings demonstrate the possible existence of a novel cellular pathway that involves physical interaction between EGFR and DNA-PK or Ku70/80 in response to blockade of EGFR signaling by specific mAbs.

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