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

(Received for publication, September 22, 1997, and in revised form, November 14, 1997)

Debdutta Bandyopadhyay‡, Mahitosh Mandal‡, Liana Adam‡, John Mendelsohn, and Rakesh Kumar§

From the Cell Growth Regulation Laboratory, Department of Clinical Investigation, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

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 EGFR1 induced by extra-

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.

* This work was supported in part by American Institute for Cancer Research Grants 94B93 and 96A077, National Institutes of Health Grant CA65746, and new research program funds from The University of Texas M. D. Anderson Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this study.

§ To whom correspondence should be addressed: Cell Growth Regulation Section, The University of Texas M. D. Anderson Cancer Center (Box 36), 1515 Holcombe Blvd., Houston, TX 77030. E-mail: rkumar@notes.mdacc.tmc.edu.

1568 This paper is available on line at http://www.jbc.org
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), 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.). 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 Neo- marker Inc. (Fremont, CA).

For preparation of cell extracts, cells 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 mM NaVO₅, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. The lysate was centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. 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 amount of cell lysates were metabolically labeled for 6 or 3 h with 100 μCi/ml [35S]methionine in methionine-free medium containing 2% fetal bovine serum in the absence or presence of mAb 225. Alternatively, cells were labeled for 3 h and treated with mAb for the last 30 min of culture before harvesting. Cell 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-PKcs subunits Ku70/80 mAbs, and EGFR kinase activity was measured by an 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 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. After 10 min and 10 minutes 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 mM 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 assays using the DNA-PK kinase activity, cytosolic and nuclear fractions (35) were prepared and assayed using a peptide substrate (EPPLSQEAFAADLWKK, purchased from the Promega) specific for DNA-PK as per the manufacturer’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 and permeabilized in acetone at −20 °C for 30 min. Several dilutions of each antibody were used to optimize the final staining. For DNA-PK staining, DiFi cells were treated with or without anti-DNA-PK mAb 225–4 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 anti-EGFR mAbs and not in response to EGFR ligands. Regulat
Regulatory Interactions between EGF Receptor and DNA-PK

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 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. 3A, lanes 2 and 5). Data in Fig. 3B 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. 4A, lanes 1 and 2). The EGFR tyrosine kinase displayed intrinsic activity in the absence of DNA-PK (lane 1) or Ku70/80 (lane 2). The association of EGFR with DNA-PK was also established by stripping the above blots and re-immunoblotting with an 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. 3A, lanes 2 and 5). Data in Fig. 3B 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 an 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. 4A, lanes 1 and 2). The EGFR tyrosine kinase displayed intrinsic activity in the absence of DNA-PK (lane 1) or Ku70/80 (lane 2). The association of EGFR with DNA-PK was also established by stripping the above blots and re-immunoblotting with an anti-EGFR-mAb H11 (Ref. 41, raised against extracellular domain of EGFR) (data not shown).
treatment upon the status of DNA-PK phosphorylation in these assays (Fig. 4A, lanes 3–5). Subsequent to autoradiography, the identity of the phosphorylated protein bands was confirmed by immunoblotting with specific Abs against DNA-PK or EGFR (Fig. 4B). 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. 4C 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. 4D). 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.
widely considered as potential antiproliferative 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 EGFR.

cells, and treatment with mAb 225 was accompanied with 75% reduction in the levels of the nuclear DNA-PK activity. Data in Fig. 5D 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. 6D, red staining). Results in Fig. 6E 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 antiproliferative therapeutic
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 does not result 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 result 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.

Acknowledgment—We thank Joseph Schlessinger for HER14 cells and RK-2 antibody.

REFERENCES
1. Yarden, Y., and Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443–478
2. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
3. Aaronson, S. A. (1991) Science 254, 1146–1153
4. Carpenter, G. (1987) Annu. Rev. Biochem. 56, 881–914
5. Kumar, R., and Mendelsohn, J. (1991) Curr. Opin. Oncol. 3, 70–74
6. Reidel, H., Massoglia, S., Schlessinger, J., and Ullrich, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1477–1481
7. Mendelsohn, J. (1990) Semin. Cancer Biol. 1, 339–344
8. Gill, G. N., Kawamoto, T., Cochet, C., Le, A., Sat2, J. D., Masui, H., MacLeod, C. I., and Mendelsohn, J. (1984) J. Biol. Chem. 259, 7755–7760
9. Kawamoto, T., Sat2, J. D., Le, A., Polikoff, J., Sat2, G. H., and Mendelsohn, J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1337–1341
10. Van de Vijver, M. J., Kumar, R., and Mendelsohn, J. (1991) J. Biol. Chem. 266, 7503–7508
11. Atlas, I., Mendelsohn, J., Baserga, J., Fair, W. R., Masui, H., and Kumar, R. (1992) Cancer Res. 52, 3335–3339
12. Fan, Z., Mendelsohn, J., Masui, H., and Kumar, R. (1993) J. Biol. Chem. 268, 21073–21079
13. Fan, Z., Lu, Y., Wu, X., and Mendelsohn, J. (1993) J. Biol. Chem. 269, 25795–25802
14. Fan, Z., Lu, Y., Wu, X., DeBiasio, A., Koff, A., and Mendelsohn, J. (1995) J. Cell Biol. 131, 235–242
15. Wu, X., Fan, Z., Masui, H., Rosen, N., and Mendelsohn, J. (1995) J. Clin. Invest. 95, 1897–1905
16. Honegger, A. M., Dull, T. J., Felder, S., Oberghen, E. V., Bellet, F., Szapary, D., Schmidt, Ullrich, A., and Schlessinger, J. (1987) Cell 51, 199–209
17. Baserga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W. M., Jr., and Mendelsohn, J. (1993) J. Natl. Cancer Inst. 85, 1327–1333
18. Fan, Z., Baserga, J., Masui, H., and Mendelsohn, J. (1993) Cancer Res. 53, 4637–4642
19. Mendelsohn, J., and Fan, Z. (1997) J. Natl. Cancer Inst. 89, 341–343
20. Carter, T., Vancurova, I., Sun, I., Lou, W., and DeLeon, S. (1996) Mol. Cell. Biol. 16, 6460–6471
21. Jackson, S. P., and Jeggo, P. A. (1995) Trends Biochem. Sci. 20, 412–415
22. Weaver, D. T. (1995) Trends Genet. 11, 388–392
23. Reeves, W. H., Wang, J., Ajmani, A. K., Stojanov, L., and Satoh, M. (1997) in The Antibodies (Zanetti, M., and Capra, J. D., eds) Vol. 3, pp. 33–84, Harwood Academic Publishers, Amsterdam, Netherlands
24. Chan, D. W., and Lees-Miller, S. P. (1996) J. Biol. Chem. 271, 8936–8941
25. Haimovitz-Friedman, A., Vlodavsky, I., Chaudhuri, A., Witte, L., and Fuks, Z. (1991) Cancer Res. 51, 2552–2558
26. Mandal, M., Wu, X., and Kumar, R. (1997) Carcinogenesis 18, 229–232
27. Mandal, M., and Kumar, R. (1997) J. Biol. Chem. 272, 14183–14187
28. Korutla, L., Cheung, J. Y., Mendelsohn, J., and Kumar, R. (1995) Carcinogenesis 16, 1741–1745
29. Kumar, R., Shepard, H. M., and Mendelsohn, J. (1991) Mol. Cell. Biol. 11, 979–986
30. Kumar, R., Horita, L., and Zang, K. (1994) J. Biol. Chem. 269, 25437–25441
31. Kumar, R., and Atlas, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6599–6603
32. Mandal, M., Maggirwar, S. B., Sharma, N., Kaufmann, S. H., Sun, S. C., and Kumar, R. (1996) J. Biol. Chem. 271, 30354–30359
33. Korutla, L., and Kumar, R. (1994) Biochim. Biophys. Acta 1224, 597–600
34. Sun, S. C., Maggirwar, S. B., and Harbaj, E. (1995) J. Biol. Chem. 270, 18347–18351
35. Peterson, S. R., Kurimasa, A., Oshimura, M., Dynan, W. S., Bradbury, E. M., and Chen, D. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3117–31174
36. Lees-Miller, S. P., Sakaguchi, K., Ulrich, S. J., Appela, E., and Anderson, C. W. (1992) Mol. Cell. Biol. 12, 5041–5049
37. Chan, C. W., and Lees-Miller, S. P. (1996) J. Biol. Chem. 271, 8936–8941
38. Martin, T. E., Barghusen, S. C., Leser, G. P., and Spear, P. G. (1987) J. Cell Biol. 106, 2069–2082
39. Morrison, P., Chung, K.-C., and Rosner, M. R. (1996) Biochemistry 35, 14618–14624
40. Kris, R. M., Lux, I., Glick, W., Waterfield, M. D., Ullrich, A., Fridkin, M., and Schlessinger, J. (1985) Cell 40, 619–625
41. Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurud, S. N., McLendon, R. E., Muscatelli, D., Peggam, C. N., Reist, C. J., Traweek, S. T., Wong, A. J., Zalutsky, M. R., and Bigner, D. D. (1995) Cancer Res. 55, 3140–3148