Radiation-induced Epidermal Growth Factor Receptor Nuclear Import Is Linked to Activation of DNA-dependent Protein Kinase*

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Ionizing radiation, but not stimulation with epidermal growth factor (EGF), triggers EGF receptor (EGFR) import into the nucleus in a probably karyopherin α-linked manner. An increase in nuclear EGFR is also observed after treatment with H2O2, heat, or cisplatin. During this process, the proteins Ku70/80 and the protein phosphatase 1 are transported into the nucleus. As a consequence, an increase in the nuclear kinase activity of DNA-dependent kinase (DNA-PK) and increased formation of the DNA end-binding protein complexes containing DNA-PK, essential for repair of DNA-strand breaks, occurred. Blockade of EGFR import by the anti-EGFR monoclonal antibody C225 abolished EGFR import into the nucleus and radiation-induced activation of DNA-PK, inhibited DNA repair, and increased radiosensitivity of treated cells. Our data implicate a novel function of the EGFR during DNA repair processes. The epidermal growth factor receptor (EGFR) is essential for mediation of both proliferative and survival signals to cells (1). At least five mitogenic growth factors bind to and activate EGFR. In addition to EGF these factors include transforming growth factor-α, amphiregulin, heparin binding EGF (2), and epiroregulin (3). Moreover, in recent years it became apparent that in addition to ligand-binding-induced activation of the EGFR, ligand-independent receptor activating processes also exist (4). Activation of the EGFR signaling pathway by ligands has been linked with decreased apoptosis (5). As a consequence it is assumed that increased EGFR signaling plays an important role in tumorigenesis (6, 7). The mechanisms of ligand-independent EGFR activation and also the importance for the cell fate are not understood so far. But it is noteworthy that the ligand-independent activation always is linked to exposure to genotoxic stress (4, 8, 9). However, the differences of the observed cell response after ligand-induced EGFR activation, e.g. cell proliferation, and ligand-independent activation, e.g. cell cycle arrest, argue rather for a link to DNA repair processes for ligand-independent activation. This idea was strengthened by the observation that many tumor cells show an increased radiosensitivity after inhibition of EGFR signaling (10). The most detrimental DNA damages after treatment with ionizing radiation are double-strand breaks (11), which are preferentially repaired in mammals by nonhomologous end-joining (12). Looking for a connection between radiation-induced EGFR activation and DNA repair, we found a report stating that there is a physical interaction of EGFR and DNA-dependent kinase (DNA-PK) (13) after cell treatment with the EGFR blocking antibody C225. As a consequence, the nuclear DNA-PK protein and activity was reduced, providing an explanation for the radiosensitizing effect of the EGFR blockage. However, this paper did not answer the question of the functional role of the physical interaction between EGFR and DNA-PK. Therefore, we addressed herein the question of the role of the complex formation between EGFR and DNA-PK and the importance for cell survival after exposure to ionizing radiation. We could show that EGFR import to the nucleus proceeds the complex formation in the nucleus. The EGFR import and the linked transport of accessory proteins leads to an increase in DNA-PK activity, which correlates to an increased formation of the DNA end-binding protein complex. Thus, these data directly link the ionizing-induced activation of the EGFR with DNA repair processes and give an explanation for the success of combination of radiation treatment and EGFR blockage during tumor treatment.

There is increasing evidence that overexpression or mutation of EGFR is associated with aggressive tumor growth as well as with poor treatment outcome of common cancers in man (14). This association is likely related to the EGFR essential role in mediating both proliferative and survival signals in cells (1). Exposure to genotoxic stress results in ligand-independent phosphorylation and activation of the EGFR (4). It is speculated that this process is linked with regulation of DNA repair processes (10, 15).

Two recent observations encouraged us to focus our interest on a possible link between EGFR and DNA repair. First, Banypash PD et al. (13) showed a physical interaction between EGFR and the DNA repair relevant enzyme (DNA-PK) (11) after incubation with the EGFR-blocking antibody C225. Second, Lin et al. (16) demonstrated nuclear localization of EGFR which would bring the EGFR in physical contact with sites of...
DNA damage. Thus, we analyzed the location of EGFR and its molecular interactions after radiation exposure.

MATERIALS AND METHODS

Cell Culture and Irradiation—Human bronchial carcinoma cells, designated A549 (17), were used in all experiments. Additionally, cells of the human squamous carcinoma cell line FaDu (ATCC, origin head and neck cancer, passage 124) and of the transformed human fibroblast cell line HH4dd (derived from human skin, passage 65–70) were used. The HH4dd cell line arose spontaneously from the normal cell strain HH4 and is characterized by growth in soft agar, aneuploidy, and tumor growth in nude mice (18). Confluent cultures were irradiated with 225-kV photons (Gulmay R S225) at a dose rate of 3 Gy/min at 37 °C. Trypsinized cells were seeded for colony formation assay in 75-cm² plates. 24 h after plating cells were irradiated, and after 10 days colonies were fixed, stained, and counted. EGFR antibody C225 (Cetuximab) was purchased from Oncogene (Boston, MA) and used in a concentration of 30 ng/ml for 1 h before irradiation.

Confocal Microscopy—A549 cells were cultivated on CultureSlides (BD Biosciences), irradiated with 4 Gy, and after 5 min fixed with peroxidate-lysine-paraformaldehyde. For immunofluorescence analysis, cells were incubated with anti-EGFR (BD Biosciences, clone 13) (1:20) overnight at 4 °C. Bound antibodies were visualized by incubation with a 1:500 dilution of a Cy3-donkey anti-mouse serum (Dianova, Hamburg, Germany) for 1 h. Nuclei were stained with YO-PRO (Molecular Probes, Leiden, The Netherlands). The nuclei were analyzed with a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems, Bensheim, Germany).

Detection of EGFR-specific Immune Complexes—Confluent cell cultures were irradiated as described above, and immune precipitation was performed according to standard procedures with the anti-EGFR antibody (clone R19/48, BIOSOURCE) (1:200) overnight at 4 °C. Western blotting was performed according to standard procedures. The primary antibodies were diluted as follows: anti-DNA-PK (Pharmingen, clone 4F10C5) 1:500, phospho-specific T2609 antibody-DNA-PK (both generous gifts of Benjamin Chen), anti-EGFR (BD Transduction Laboratories, clone 13) 1:1000, anti-P-EGFR (Calbiochem, clone 9H2) 1:1000, anti-Ku 70 (BD Transduction Laboratories, clone 15) 1:500, anti-Ku 80 (BD Transduction Laboratories, clone 111) 1:500, anti-protein phosphatase 1-phosphatase (Santa Cruz Biotechnology, clone FL-18) 1:1000, anti-phosphoryrosyne (Oncogene, clone PY20) 1:250, and calnexin (BD Transduction Laboratories, clone 37) 1:500. Quantification was achieved by incubation with a secondary peroxidase-conjugated antibody with the ECL Plus system (Amersham Biosciences).

EGFR Autophosphorylation—Confluent cells were treated with ionizing radiation and cells then lysed, and the EGFR was immunoprecipitated as described above. Immunoprecipitates werebuffered with 10 mM Hepes, pH 7.5, 50 mM glycerophosphate, 50 mM NaCl, 10 mM dithiothreitol, 1% Triton X-100, 10 μM ATP. Kinase activity was started by the addition of 1 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) for 30 min at 30 °C. Subsequently, the reaction was stopped by the addition of SDS loading buffer. Proteins were electrophoretically separated by SDS-polyacrylamide gel electrophoresis. EGFR autophosphorylation was visualized by fluorography.

Subcellular Fractionation—Cytoplasmic and nuclear extracts were prepared according to the instructions of the NE-PER® nuclear and cytoplasmic extraction reagents (Pierce). Nuclear extracts (10 μg) were incubated with the 32P-labeled double-stranded oligonucleotide GGGCCAAAGAATCTTAGCAGTTTCGGG (21). The gel shift assay was performed according to procedures described in technical bulletin no. 110 (Promega, Madison, WI). The binding reaction was performed in AP1 binding buffer (Promega) for 20 min at room temperature. For the supershift reaction, 10 μg of nuclear extract was preincubated with 5 μg of DNA-PK antibody (RB-DPK10, GeneTex, San Antonio, TX) for 2 h at room temperature before the DNA end binding reaction.

Quantification of γ-H2AX Foci Formation—Cells were cultivated on CultureSlides (BD Biosciences), incubated with C225 (30 ng/ml) for 1 h, irradiated, and fixed with 70% ice-cold ethanol after 24 h. For immune-fluorescence analysis cells were incubated with γ-H2AX antibody (Upstate Biotechnology, Lake Placid, NY, clone JBW301) (1:500) for 2 h at room temperature. Positive foci were visualized by incubation with a 1:500 dilution of Alexa-488-labeled goat anti-mouse serum (Molecular Probes) for 30 min. Coverslips were mounted in Vectashield/4,6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, UK). For each data point from 300 to 500 nuclei were evaluated.

RESULTS

To elucidate the subcellular localization of EGFR after irradiation, we performed confocal microscopy. We failed to detect EGFR in the nucleus of unirradiated proliferating cells of the bronchial carcinoma line A549 in a noteworthy amount (Fig. 1, A and C). But there was a strong signal for EGFR in the perinuclear cell compartment. Irradiation, however, resulted in a clear translocation of the perinuclear EGFR to the nucleus (Fig. 1, B and D), and EGFR was detectable predominantly within regions characterized by sparse chromatin condensation (Fig. 1, D and E). Nuclear fluorescence intensity profiles integration indicated that irradiation increased Cy3 intensity (EGFR staining) within the nuclear region up to 24 times (data not shown).
A lamin B1 protein remained constant. The used stressors belong to the group of DNA-damaging agents, which suggests that the nuclear transport of the EGFR may be related to DNA repair processes.

To determine the role of nuclear EGFR import, the physical interaction between EGFR and DNA-PK was investigated. Cells were exposed to a single dose of 4 Gy of ionizing radiation. Subsequently, immune precipitation of EGFR and DNA-PK was performed. In all three cell lines tested (A549, HH4dd, and FaDu), we detected a rapid induction of EGFR phosphorylation within 5 min after treatment. The phosphorylated EGFR in the cytoplasm and nucleus after irradiation was measured after 5 and 10 min. Western blotting showed that EGFR phosphorylation increased in the nucleus, reaching a maximum at 20 min. At this time point, cytoplasmic EGFR phosphorylation had decreased, but it increased in the nucleus, reaching a maximum at 20 min. This time point cytoplasmic EGFR phosphorylation had returned to basal level.

To further elucidate the function of nuclear EGFR, A549 cells were treated with several cellular stressors, and time-dependent nuclear EGFR import was quantified (Fig. 3B). Incubation with either H2O2 or cisplatin as well as treatment with heat (42 °C) induced EGFR transport into the nucleus within 5 min with similar time kinetics, whereas the amount of nuclear lamin B1 protein remained constant. The used stressors belong to the group of DNA-damaging agents, which suggests that the nuclear transport of the EGFR may be related to DNA repair processes.

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**FIG. 2. Detection of the EGFR in the nucleus of irradiated or EGF-treated cells.** A, A549 cells were irradiated with 4 Gy, and cytoplasmic and nuclear proteins were isolated. In the cytoplasmic fraction EGFR and the ER-specific protein calnexin were quantified by Western blotting (WB). From the nuclear fraction DNA was extracted, and bound proteins were separated by SDS-PAGE. After blotting, EGFR and calnexin were detected by specific antibodies. Lamin B1 was detected in an aliquot of nuclear fraction. B, the experiment was performed as described for A; however, cells were treated with EGF (100 ng/ml). Each experiment was performed at least three times; shown are representative results.

To confirm the nuclear localization of the EGFR, we separated cell cytoplasm and nuclei after irradiation. DNA-bound proteins in the nuclear fraction were cross-linked to DNA by incubation with formaldehyde, and subsequently DNA was precipitated by the addition of isopropyl alcohol. Within this preparation of DNA-bound proteins, we detected EGFR in increasing amounts with time after irradiation (Fig. 2A), whereas lamin B1 protein did not change. To exclude the possibility that ER-protein contaminants produced artifacts, we tested for the presence of calnexin, an ER-marker protein, in this preparation. We failed to detect calnexin within the fraction of DNA-bound proteins but did detect it within the fraction of cytoplasmic proteins, where it was abundant (Fig. 2A).

To determine whether nuclear EGFR import also occurs after ligand-dependent EGFR activation, we treated cells with EGF. In contrast to the situation after irradiation, only a small amount of EGFR was detectable in the fraction of DNA-bound proteins both before and after treatment with EGF (Fig. 2B). We concluded from these results that nuclear import of EGFR occurs mainly after ligand-independent activation of EGFR by irradiation.

The EGFR is phosphorylated both in a ligand-dependent and -independent manner. Thus, we asked the question of whether this phosphorylation can be linked with the observed nuclear transport. In A549 carcinoma cells, we detected a rapid increase in cytoplasmic EGFR phosphorylation within 5 min after irradiation (Fig. 3A). A rapid induction of EGFR phosphorylation was also observed after EGF treatment (Fig. 3A). With a short delay, EGFR phosphorylation increased in the nucleus as well, exclusively when cells were exposed to ionizing radiation. With increasing time after irradiation (from 5 to 20 min), EGFR phosphorylation in the cytoplasm decreased, but it increased in the nucleus, reaching a maximum at 20 min. At this time point cytoplasmic EGFR phosphorylation had returned to basal level.

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To further elucidate the role of EGFR/DNA-PK complex formation we tested for an effect upon DNA-PK activity. Therefore, we irradiated cells and then assayed them for DNA-PK activity in the nucleus and the cytoplasm. The major activity was detected within the nucleus. This activity significantly increased within 5 min after irradiation, as quantified by phosphorylation of a synthetic TP53-derived peptide (Fig. 3D).

Using several cell lines and strains, we could show that radiation-induced EGFR nuclear transport can be correlated with DNA-PK activity (Fig. 3E). This is not only true for cells presenting an average radiosensitivity profile, as determined by clonogenic assays, e.g. the bronchial carcinoma cell line A549 (SF2 = 0.59) and normal skin fibroblasts (SF2 = 0.55), but also for FaDu cells, which are classified as radio-resistant (SF2 = 0.7). In contrast, in HL60 cells (SF2 = 0.34), which are EGFR-deficient, radiation failed to induce an EGFR transport into the nucleus, and the basal activity of DNA-PK is rather decreased after irradiation. Whereas in highly radiosensitive SCID cells (SF2 = 0.1), deficient in DNA-PK function, the radiation-induced EGFR transport to the nucleus was functional, but no DNA-PK activity was induced (Fig. 3E).

We performed additional experiments to address a possible role for the import of EGFR and associated proteins to the nucleus in regulation of DNA-PK. First we determined the levels of EGFR protein in both the cytoplasm and the nucleus after irradiation (Fig. 4A). Shortly after irradiation, the EGFR levels in the nucleus increased (Fig. 4A). After the nuclear import of the EGFR increased, complex formation between DNA-PK and EGFR was observed (Fig. 4B). Moreover, the import of EGFR to the nucleus was accompanied by increased appearance of Ku 70 and Ku 80 proteins in the nucleus (Fig. 4, A and D), involved in regulation of DNA-PK activity.

As known so far, DNA-PK activity is regulated by phosphorylation (20) and dephosphorylation (22) events. Phosphorylation at specific sites results in inactivation of enzyme activity; however, activity can be restored by protein phosphatase 1 or protein phosphatase 2A enzymes (23). We found that an increase in protein phosphatase 1 in the nucleus after irradiation was associated with the transport of the EGFR protein complex to the nucleus (Fig. 4E). To prove purity of nuclear protein preparation, we tested for contaminations with aldolase, a cytoplasmic marker protein. We could show that within the nu-

**Fig. 4. Import of the EGFR-complex into nucleus after irradiation.** Bronchial carcinoma cells (A549) were grown to confluence and irradiated with 4 Gy. At the time points 0, 5, 10, and 20 min after irradiation cells were lysed, and cytoplasmic and nuclear proteins were isolated. The protein complexes were dissolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The proteins EGFR (A), DNA-PK (B), Ku70 (C), Ku80 (D), and protein phosphatase 1 (PP1; E) were detected with help of specific antibodies. The success of the cytoplasmic and nuclear protein separation was proved by detection of the cytoplasmic protein aldolase and the nuclear protein histone H1 (F). Each experiment was performed at least three times; shown are representative results. IP, immunoprecipitated; WB, Western blot.

**Fig. 5. Molecular mechanism of the EGFR import into the nucleus.** A549 cells were treated with β-methylcyclodextrin (20 mM) (A), an inhibitor of coated pits formation, for 30 min, and subsequently cells were irradiated. Nuclear proteins were isolated, and the EGFR protein amount was quantified after Western blotting (WB). As the loading control lamin B was quantified. B, the experiment was performed as described for A, but cells were treated with the radical scavenger acetylcysteine. C, cells were treated either with leptomycin B, an inhibitor of nuclear export, or with wheat germ agglutinin (WGA), which inhibits karyopherin-dependent nuclear import. After irradiation, nuclear proteins were isolated, EGFR was immune-precipitated (IP), proteins were separated by SDS-PAGE, and EGFR was detected. Aliquots of nuclear protein used for immune precipitation were used for lamin B1 quantification. D, cells were irradiated, nuclear proteins were isolated, the EGFR was immune-precipitated, and the protein complexes were dissolved by SDS-PAGE. After blotting, EGFR-bound proteins karyopherin α and Ran were detected by specific antibodies. Aliquots of nuclear protein used for immune precipitation were used for lamin B1 quantification. Each experiment was performed at least three times; shown are the representative results.
clear protein preparation no detectable contamination with aldolase was apparent (Fig. 4F), whereas the nuclear marker histone H1 is abundantly detectable.

To answer the question of whether nuclear EGFR is linked to cytoplasmic EGFR, we incubated cells with β-methyl-cyclohex-trin, which blocks the formation of the coated pits essential for EGFR internalization and quantified radiation-induced nuclear transport of the EGFR. Blockage of coated pit-mediated EGFR internalization had no effect on EGFR nuclear transport (Fig. 5A). Because it is described (24) that cellular stress can result in increased nuclear import by radical dependent processes, we incubated cells with the radical scavenger acetyl-cysteine and tested for radiation-induced nuclear EGFR import. We observed in the presence of acetyl-cysteine a complete inhibition of radiation-induced EGFR nuclear import (Fig. 5B).

To elucidate the mechanism responsible for the nuclear transport of the EGFR, we incubated cells with leptomycin B, a nuclear export inhibitor, and with wheat germ agglutinin, an import inhibitor through the nuclear pore. Wheat germ agglutinin treatment completely blocked the radiation-induced increase of EGFR in the nucleus, whereas leptomycin B incubation resulted in an increased amount of EGFR in the nucleus which cannot be further increased by irradiation (Fig. 5C). The transport of proteins through the nuclear pore is typically mediated by karyopherins and the protein RAN. Immune precipitation of nuclear EGFR after irradiation revealed a complex formation with both with karyopherin α and with RAN. With increasing amounts of EGFR in the nucleus, complex formation with karyopherin α and Ran also increased (Fig. 5D).

To confirm the importance of radiation-induced EGFR transport into nucleus for regulation of DNA-PK activity, we treated cells with the anti-EGFR antibody, C225, shown to block EGFR function (25). Our results presented in Fig. 6A clearly show that radiation strongly increased DNA-PK activity, which was nearly completely abolished by adding C225 antibody to cells 1 h before irradiation. To further characterize the effect of a C225 pretreatment, we incubated cells with C225 and quantified the amount of nuclear EGFR. We could show that radiation-induced nuclear EGFR transport was blocked (Fig. 6B). To further prove an effect of EGFR on regulation of DNA-PK activity, we treated A549 cells with C225 antibody (30 nm) and irradiated with 4 Gy. DNA-PK was immune-precipitated (IP) from whole cell lysates, and kinase activity was determined. DNA-PK protein was quantified by Western blotting (WB). The experiment was performed as described for A, but nuclear proteins were isolated, and EGFR was quantified after Western blotting. The nuclear protein lamin B1 was quantified as the loading control. C, A549 cells were treated with unspecific IgG or C225 antibody for 1 h (30 nm). Nuclear proteins were isolated and separated by SDS-PAGE, and after Western blotting DNA-PK and DNA-PK phosphorylation at Ser-2056 were detected. D, the experiment was performed as described for C, but DNA-PK phosphorylation at Thr-2609 was quantified with a specific antibody. Each experiment was performed at least three times; shown are representative results.

FIG. 6. Regulation of radiation-induced DNA-PK activity and its link to EGFR. A, role of EGFR import for the radiation induced DNA-PK activation. A549 cells were pretreated with C225 antibody (30 nm) or mouse IgG (30 nm) as a control for 1 h and subsequently irradiated with 4 Gy. DNA-PK was immune-precipitated (IP) from whole cell lysates, and kinase activity was determined. DNA-PK protein was quantified as the loading control. B, the experiment was performed as described for A, but nuclear proteins were isolated, and EGFR was quantified after Western blotting. The nuclear protein lamin B1 was quantified as the loading control. C, A549 cells were treated with unspecific IgG or C225 antibody for 1 h (30 nm). Nuclear proteins were isolated and separated by SDS-PAGE, and after Western blotting DNA-PK and DNA-PK phosphorylation at Ser-2056 were detected. D, the experiment was performed as described for C, but DNA-PK phosphorylation at Thr-2609 was quantified with a specific antibody. Each experiment was performed at least three times; shown are representative results.

To estimate the relevance of DNA-PK activity and the functional consequences of the C225-induced inhibition of nuclear EGFR transport and DNA-PK complex formation, we assessed the formation of the DNA end binding complex (Fig. 7). Using an electrophoretic mobility shift assay, we identified two protein complexes whose expression was increased by irradiation. Treatment with C225 antibody inhibited formation of these complexes. Both complexes contained DNA-PK, as determined by supershift experiments using DNA-PK antibodies.

To find out whether inhibition of radiation-induced DNA-PK activity can be correlated with decreased DNA repair after irradiation, we quantified yH₂AX foci formation, which marks DNA strand breaks (26, 27) in the presence of C225 antibody (Fig. 8). Simultaneously we determined clonogenic survival by colony formation assays. We observed a slowed kinetic of yH₂AX-foci removal, which corresponds to a slowed DNA damage repair (Fig. 8A). Most noteworthy, residual damage 24 h after irradiation with different radiation doses was signifi-

FIG. 7. Quantification of DNA end binding complex formation. A549 were pretreated with the antibody C225 (30 nm) for 1 h and irradiated with 4 Gy. Nuclear proteins were isolated. Nuclear extracts (10 μg) were incubated with the ³²P-labeled double-stranded oligonucleotide GGCCCAGGATCTTAGCAGTTTCCGGG, and a gel shift assay was performed. C, competition with an unlabeled DNA-oligo. For the supershift reaction the DNA-PK antibody (Ab) (RB-DPK10, GeneTex) was used.

Interestingly, pretreatment of cells with C225 did not effect autophosphorylation of DNA-PK at Ser-2065 (Fig. 6A). Because it is described (24) that cellular stress can result in increased nuclear import by radical dependent processes, we incubated cells with the radical scavenger acetyl-cysteine and tested for radiation-induced nuclear EGFR import. We observed in the presence of acetyl-cysteine a complete inhibition of radiation-induced EGFR nuclear import (Fig. 5B).

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Recently the EGFR has been detected in the nucleus of various tissues and cell lines (16). Based on these data the authors suggest a role of EGFR as transcription factor. Moreover, based on data reporting a physical interaction between the EGFR and DNA-PK in mammalian cells (13), we addressed the potential role during cellular stress response, i.e. regulation of DNA repair after DNA damage. Consequently, as a major point we had to show that the EGFR is responsive upon irradiation and cellular stress. Indeed, it has been reported for some time that EGFR is phosphorylated after cellular stress responses, i.e. irradiation (28, 29). However, the biological role of this process and the linked stress-associated intracellular signaling has to be elucidated. We could clearly show that after irradiation the EGFR enters the nucleus. This was shown by help of confocal microscopy, immune detection in nuclear preparations, and after DNA precipitation experiments. Thus, we are convinced that the EGFR is localized in the nucleus under certain circumstances, although we are aware to the possibility of a contamination, since the EGFR is ER-associated and the ER membrane is contiguous with the nuclear membrane, which means that protein localized to the ER could easily be misinterpreted as nuclear localization (30). Nevertheless, we could clearly show that the EGFR was detectable mainly in regions with low DNA density (Fig. 1, D and E). This observation fits perfectly with the idea that EGFR either interacts with DNA repair processes, since DNA repair preferentially occurs in regions of sparse chromatin condensation, where the access to DNA damage is eased, or acts as a stress-induced transcription factor (16).

As already mentioned the EGFR import is accompanied by a phosphorylation of the EGFR induced by ionizing radiation in a ligand-independent manner. Also, after a ligand-dependent activation with EGF the EGFR is found to be phosphorylated (8); however, in this case the amount of EGFR in the nucleus is not increased.

Although EGFR phosphorylation induced by EGF treatment is stronger than that induced by irradiation, the amount of EGFR in the nucleus is not increased compared with radiation treatment. Thus, one can assume that EGFR phosphorylation either does not trigger the transport of EGFR to the nucleus or there is a qualitative difference in EGFR phosphorylation, e.g. phosphorylation at different Tyr or other amino acid residues, induced by EGF or irradiation.

However, treatment with various stressors clearly increased EGFR protein in the nucleus. All stressors used have in common that they induce DNA damage, which suggests that DNA damage may be the trigger for EGFR transport into the nucleus. Because a substance like cisplatin or treatment with heat does not induce double strand breaks but the EGFR translocation is induced within minutes, it is not likely that double strand breaks are the trigger for EGFR nuclear transport. More likely, radical production or a wide spectrum of DNA damage may be the trigger for EGFR transport into the nucleus. Since EGFR is ER-associated, DNA repair may be the trigger for nuclear translocation. This hypothesis fits also the observation that the use of radical scavengers very efficiently abolished radiation-induced EGFR translocation (Fig. 5B).

In all three cell lines tested (A459, HH4dd, and FaDu), we detected increased complex formation of EGFR and DNA-PK within 5 min after radiation exposure (Fig. 3C). In the context of other data (31), this result suggests a role for EGFR in DNA-PK-mediated DNA repair and cell survival after irradiation (10).

Indeed, we detected activation of kinase function of the DNA-PK, which is obligatory for nonhomologous DNA end joining, shortly after EGFR nuclear import following irradiation. (Fig. 3D). The coincidence of appearance of EGFR in the nucleus and the increase in DNA-PK activity was observed in several cell
lines and cell strains (Fig. 3E). It is noteworthy that in EGFR-deficient cells (HL60) the radiation-induced activation of DNA-PK activity was prevented, whereas in DNA-PK-deficient cells EGFR nuclear import was functional. However, in both cases radiosensitivity was increased dramatically. This means for cell survival after radiation treatment both the EGFR nuclear import and the activation of DNA-PK are essential.

The import of EGFR to the nucleus was accompanied by increased appearance of Ku 70 and Ku 80 proteins in the nucleus (Fig. 4, C and D). Subsequently, complex formation between EGFR and DNA-PK was observed inside the nucleus. These data further support the notion that nuclear EGFR may be involved in regulating DNA-PK function. Efficient DNA repair requires formation of a complex between DNA-PK and the heterodimer of Ku 70/80 (22, 32), but the exact role of Ku 70/80 in this complex is not yet fully resolved. The presence of Ku 70/80 was reported to be required for a number of functions including binding of DNA-PK core domain to free DNA ends (33), for synthesis of DNA ends (32), and for kinase activation by DNA (34). Although the core domain of the DNA-PK possesses end binding activity already, to be maximally effective in repairing DNA, all three subunits (DNA-PK, Ku 70, and Ku 80) must be present in the complex. DNA-PK activation occurs only after DNA synthesis is completed, leading to phosphorylation of accessory proteins and to dissociation of the DNA-PK from repair complex after autophosphorylation (22, 23). DNA-PK activity, however, can be restored by protein phosphatase 1 (23), which is also a compound of the EGFR complex transported to the nucleus after irradiation. This suggests that reversible activation and inactivation is an important mechanism in regulating DNA-PK function is.

To understand the molecular mechanism of the nuclear import of the EGFR complex, we asked the question of whether the imported EGFR is directly fed from EGFR pool localized at the cell membrane, or it is derived form EGFR located perinuclear? Treatment with β-methyl cyclodextrin is sufficient to prevent internalization of the EGFR by coated pits at a concentration of 20 mM (35). However, the radiation-induced nuclear import of the EGFR was not influenced (Fig. 5A). This argues for a nuclear import of mainly perinuclear EGFR after irradiation. This conclusion fits perfectly to the observations made with confocal microscopy.

Because the EGFR and associated proteins form a large complex that needs active transport into the nucleus, we made the attempt to identify the responsible transport mechanism. Lin et al. (15) show that EGFR exhibits a putative nuclear localization sequence (16), which would allow transport by karyopherins (36). In addition Williams et al. (37) described for the EGFR-related HER4 a nuclear localization sequence and a function as a nuclear chaperone.

Indeed, we could show that the inhibition of the nuclear import by wheat germ agglutinin resulted in inhibition of radiation-induced EGFR import to the nucleus. Inhibition of the nuclear export by leptomycin, an inhibitor of chromosome region maintenance protein (CRM1) (38), resulted in an increase of basal EGFR in the nucleus, which could not be further increased by irradiation. Taking both inhibitor experiments together, the results argue for an irradiation-triggered karyopherin-dependent transport of EGFR into the nucleus, where the EGFR amount is regulated by import and export processes. This idea is confirmed by the observation that nuclear EGFR is found in a complex with karyopherin α and Ran protein, both essential for formation of a nuclear localization sequence-dependent nuclear import complex. Moreover, we observed that nuclear EGFR import is inhibited in the presence of radical scavengers, i.e. acetylcysteine. This means that radicals are involved in the induction of the transport process. Interestingly, it is reported that cellular stress is able to induce nuclear accumulation of karyopherin α (24), which we found in complex with nuclear EGFR.

To elucidate the role of nuclear EGFR, we attempted to get insights into DNA-PK activity regulation by EGFR. Indeed, we detected activation of kinase function of the DNA-PK shortly after irradiation and EGFR nuclear transport (Fig. 5). This was observed with a peptide phosphorylation assay and also in vivo by quantification of phospho-Thr-2609 and phospho-Ser-2056 of the catalytic subunit of DNA-PK, which results from autophosphorylation (20, 22, 39). The maximal activity could be detected 10 min after irradiation, which is consistent with published data. Thus, our observations suggest that DNA-PK activity is not essential for the initial steps of nonhomologous DNA end joining since the DNA repair process begins before the 10-min mark (40). Instead, DNA-PK activity is required for efficient end processing during DNA double-strand break repair (41) or has a unknown function. Neither the exact mechanism by which DNA-PK is activated in response to DNA damage nor its physiological targets have been identified specifically. Besides phosphorylation of substrates, e.g. Werner's syndrome protein (42) and replication protein A (43), autophosphorylation at several sites in the catalytic subunit of DNA-PK (22) seems to play a crucial role during formation and resolving of the DNA end binding complex essential for nonhomologous DNA end joining (41). Autophosphorylation of DNA-PK, especially at residue Thr-2609, acts as an attenuating feedback loop, which may be critical for cellular survival after exposure to ionizing radiation (44).

The most convincing data for a link between radiation-induced nuclear EGFR import and regulation of radiation-induced DNA-PK activation came from the experiments using the EGFR-specific antibody C225 (Cetuximab), which is able to block EGFR function (45). Preincubation with this EGFR-specific antibody blocked EGFR transport into the nucleus very efficiently and inhibited DNA-PK kinase activation after irradiation.

The inhibition of nuclear EGFR import by the antibody C225, which binds to the extracellular domain of the EGFR, also rebuts the proposal that the observation of nuclear localization of the EGFR is due to contamination of the nuclear preparation with newly synthesized EGFR bound to the ER during protein synthesis (30), because it shows the EGFR was membrane-localized when antibody C225 bound to its extracellular domain.

These results suggest that treatment with the antibody C225 resulted in receptor complex internalization and protein stabilization but blocked transport of the receptor complex into the nucleus. This block decreases DNA-PK activity essential for DNA repair and cell survival after irradiation (44). This observation is in agreement with our result that C225 treatment blocks phosphorylation of DNA-PK at Thr-2609 and not at Ser-2056 selectively after irradiation. It is reported that blockage of the Thr-2609 phosphorylation site abolishes DNA-PK function and is associated with increased radiosensitivity (20). This observation also fits with the data that C225 treatment stalls formation of the cellular DNA end binding protein complex after radiation. A correlation between inhibition of DNA-PK activity, reduced formation of the DNA end binding complex, and reduced cellular survival has already been reported (46). Moreover, C225-mediated EGFR blockage has been reported to enhance radiosensitivity of tumor cells in vitro (47) and tumor radio response in vivo (48). Our findings that C225 increased residual DNA damage after irradiation, and this process correlated with a decreased clonogenic survival is in
good agreement with these published data.

Taken together, in the present study we have shown for the first time that upon cell irradiation EGFR is translocated to the cell nucleus, where it is localized predominantly in regions with uncoiled chromatin (euchromatin). These regions are easily accessible for DNA repair processes after exposure to genotoxic treatments. Moreover, we showed that the EGFR import is linked to co-transport of EGFR-associated proteins such as Ku70 and Ku80 as well as protein phosphatase 1 is linked to co-transport of EGFR-associated proteins such as Ku70 and Ku80 as well as protein phosphatase 1.

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Radiation-induced Epidermal Growth Factor Receptor Nuclear Import Is Linked to Activation of DNA-dependent Protein Kinase

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