Novel Localization of the DNA-PK Complex in Lipid Rafts

A PUTATIVE ROLE IN THE SIGNAL TRANSDUCTION PATHWAY OF THE IONIZING RADIATION RESPONSE

Hector Lucero‡, Darren Gae§, and Guillermo E. Taccioli¶

From the Departments of §Molecular and Cellular Biology, Goldman School of Dental Medicine, and ¶Department of Microbiology, School of Medicine, Boston University, Boston, Massachusetts 02118

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Increased sensitivity to ionizing radiation (IR) has been shown to be due to defects in DNA double-strand break repair machinery. The major pathway in mammalian cells dedicated to the repair of DNA double-strand breaks is by the nonhomologous end-joining machinery. Six components function in this pathway, of which three (Ku70, Ku86, and DNA-PKcs) constitute a protein complex known as DNA-dependent protein kinase (DNA-PK). However, it is now recognized that the cellular radiation response is complex, and radiosensitivity may also be regulated at different levels in the radiation signal transduction pathway. In addition to DNA damage, exposure to IR triggers intracellular signaling cascades that overlap with pathways initiated by ligand engagement to a receptor. In this study, we provide evidence for the novel localization of the DNA-PK complex in lipid rafts. We also show this property is not a generalized characteristic of all DNA repair proteins. Furthermore, we have detected Ku86 in yeast lipid rafts. Our results suggest that the components of this complex might be recruited separately to the plasma membrane by tethering with raft-resident proteins. In addition, we found an irradiation-induced differential protein phosphorylation pattern dependent upon DNA-PKcs in lipid rafts. Thus, we speculate that another role for the DNA-PKcs subunit and perhaps for the holoenzyme is in the signal transduction of IR response.

One of the most damaging lesions that can occur in a cell is a DNA double-strand break (DSB),1 because it disrupts both strands of the DNA molecule. The importance of rescuing the cell from this lesion is evident by the existence of evolutionarily conserved DNA repair systems that act upon DSBs that are generated under physiological conditions, such as during transposition, meiosis, and recombination, or by external insults like ionizing irradiation (IR) exposure. The major pathway in mammalian cells dedicated to the repair of DSBs is by the nonhomologous end-joining machinery (NHEJ). Six components function in this pathway, of which three (Ku70, Ku86, and DNA-PKcs) constitute a protein complex, termed DNA-dependent protein kinase (DNA-PK) (for reviews, see Refs. 1–4). Characterization of the DNA-PK catalytic subunit (DNA-PKcs) has revealed that it is a Ser/Thr protein kinase, a member of the phosphatidylinositol 3-kinase superfamily, and that it must be bound to DNA in order to be activated. Other protein components of the NHEJ pathway are XRC4 (5), DNA-Lig IV (6, 7), and Artemis (8). There is additional evidence for further functions either for the NHEJ or for roles of the component proteins separate from their function in NHEJ (4, 9–22).

It is now becoming clear that lipid microenvironments on the cell surface, known as detergent-insoluble glycolipid-enriched complexes (DIGs) or lipid rafts, also take part in signal transduction processes. This lipid environment favors specific protein-protein interactions between ligands, receptors, and kinases, resulting in the activation of signaling cascades (for reviews, see Refs. 39–43). Proteins incorporated into DIGs fall into different categories: glycophaspatidylinositol-anchored proteins, transmembrane proteins, doubly acylated tyrosine kinases of the Src family, and the α-subunit of heteromeric GTP-binding proteins and cholesterol linked proteins like Hedgehog (for a review, see Ref. 43).

Curiously, another member of the phosphatidylinositol 3-kinase family, which has been also associated with the response to IR, the ATM protein, has been reported to localize outside the nucleus in cytoplasmic and membrane-associated vesicles (44). Results from the same study showed that lymphoblast cells from patients carrying a mutation in the ATM gene (locus associated with a human hereditary disease known as ataxia telangiectasia) were defective in the IR-induced activation of a raft-resident protein-tyrosine kinase p53/56 Lyn.

In this study, we provide evidence for the localization of the DNA-PK complex in lipid raft of mammalian cells but not as a generalized characteristic of NHEJ factors. Furthermore, the localization of Ku86 in lipid rafts is conserved in yeast. Because the protein phosphorylation pattern after irradiation is different in lipid rafts isolated from DNA-PKcs-defective cells compared with controls, we are tempted to speculate that besides...
the role that the DNA-PK has in the DSBR pathway in mammalian, this complex might be involved in signal transduction of IR response.

EXPERIMENTAL PROCEDURES

Materials—The protease inhibitors leupeptin, aprotinin, bestatin, and Pefabloc™ were purchased from Roche Applied Science. Density gradient centrifugation medium Optiprep™ was purchased from Nycomed Pharma AS (Oslo, Norway). Primary antibodies against Ku86, Ku70, Lyn, MSH2, MSH6, XRCC4, and caveolin-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); antibodies against DNA-PKcs were from Kamiya Biomedical Co.; antibodies against Na+/K+-ATPase β1 subunit were from Upstate Biotechnology, Inc. (Lake Placid, NY); antibodies against α-tubulin were from Sigma; antibodies against calnexin were from Stressgen; and antibodies against BLM were from Serotec.

Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were purchased from Amersham Biosciences, and antibodies against goat IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Monoclonal antibody generated in rat and directed against the C-terminal part of yeast Ku86 was a gift from Dr. Heidi Feldmann (Institut fuer Biochemie der Universiteit Muenchen, Germany). All other chemicals were purchased from Sigma.

Buffers and Reagents—The following buffers were used: TNE (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA); TNEx (TNE containing 0.1% (v/v) Triton X-100); TBS (20 mM Tris-HCl, pH 7.4, 200 mM NaCl); TBST (TBS with 0.1% (v/v) Tween).

The mixture of inhibitors of proteases was as follows: 0.5 μg/ml leupeptin and aprotinin; 40 μg/ml bestatin, and 0.5 μg Pefabloc SC. The mixture of inhibitors of phosphatases was as follows: 10 mM diisopropyl pyrophosphate, 25 mM disodium β-glycerolphosphate, 1 mM sodi- dium vanadate, 50 mM sodium fluoride.

Cell Culture—The xrs-6 cell line was derived from the CHO-K1 cell line on the basis of its sensitivity to IR due to a defect in Ku86 (52). MO57J and MO57K were isolated from different areas of a glioblastoma tumor and further characterized. MO57J is radiosensitive due to a defect in DNA-PKcs as described elsewhere (50).

Cells were cultured in Dulbecco’s modified Eagle’s medium, high glucose medium (Invitrogen) supplemented with nonessential amino acids, penicillin/streptomycin, glutamine, and 10% fetal calf serum.

Preparation of Microsomal Fraction—Cells (~5 × 10^6) were detached with trypsin, washed with ice-cold PBS, and homogenized by Dounce disruption at 4°C in Tris-HCl, pH 7.5, EDTA 5 mM with protease inhibitors. Cell integrity was checked under microscope using an exclusion dye trypan blue. Before centrifugation at 700 × g, the solution was brought up to 440 mM sucrose. The postnuclear supernatant was centrifuged further, first at 12,000 × g for 15 min at 4°C to pellet mitochondria, and this supernatant was centrifuged again at 120,000 × g (Sigma). The primary antibodies were used at a 1:50 dilution for Lyn (SC 44), MO57J and MO57K were isolated from different areas of a glioblastoma tumor and further characterized. MO57J is radiosensitive due to a defect in DNA-PKcs as described elsewhere (50).

Preparation of the microsome fraction was performed on ice for 2 h at 120,000 × g for 1 h at 4°C. The pellet (microsomal fraction) was separated from the supernatant (cytosolic fraction) and resuspended in 500 μl of the above buffer containing sucrose and stored at −80°C.

RESULTS

DNA-PK Complex Is Localized in Lipid Rafts—In an attempt to unravel the role that the DNA-PK may have in the signal transduction pathway in response to IR treatment, we investigated its localization in lipid rafts or DIGs. While technical variations are employed to isolate DIGs, one set of conditions, based on the solubilization of membranes in 1% Triton X-100 at 4°C followed by flotation in sucrose or Optiprep gradients, has become the benchmark protocol within the field.

As shown in Fig. 1 (A–C), we localized components of the DNA-PK complex (Ku70, Ku86, and DNA-PKcs) in detergent-insoluble fractions, DIGs. To demonstrate that this was not a general phenomenon of members of the NHEJ machinery, a control was included for another member of this DNA repair pathway, XRCC4. As shown in Fig. 1D, the XRCC4 signal was only present in the soluble fractions. This result strongly suggests that the localization of the DNA-PK complex serves additional roles besides its main function in DSBR. Other controls included membrane hybridization with Ab against proteins reported to be present in lipid rafts like Lyn and caveolin-1, a
membrane protein Na⁺/K⁺ ATPase, which is excluded from DIGs and tubulin as a representative soluble counterpart (see Fig. 1, A and E). We have extended these studies to other gene products, which belong to other DNA repair pathways besides NHEJ. Among those, the mismatch repair genes MSH2 and MSH6, which have been localized in soluble fractions (data not shown) (45). Furthermore, the helicase BLM-1, which has been identified as the mutated target responsible for Bloom’s syndrome in humans, is restricted to soluble fractions as well (data not shown). Collectively, these results show that the localization of DNA-PK complex is not a ubiquitous event of DNA repair components.

The choice of detergent solubilization conditions is critical to the identification of DIG components. Retention of prominin in microvilli has been recently reported by using another nonionic detergent, Lubrol WX, at 4 °C, revealing a distinct cholesterol-based lipid microdomain (46). When experiments were repeated using Lubrol WX as the solubilizing detergent, results were indistinguishable from the ones achieved by using Triton X-100 (data not shown).

To fulfill one of the criteria of the operational definition of DIGs, detergent extraction was performed instead at 37 °C, and the gradient was run at room temperature. Results in Fig. 2 show, as expected, a shift to soluble fractions consistent with the identification of a protein as a component of lipid rafts.

In addition, if cholesterol is extracted by treatment with methyl-β-cyclodextrin (MCBD), raft domains are perturbed, and raft proteins usually, but not always, become detergent-soluble. Because MCBD is very toxic to the cells, treatment conditions are restricted to low concentration of this chemical to maintain cell viability. However, lipid rafts are first assembled in the Golgi, and movement out of the Golgi seems to be mainly toward the plasma membrane. Thus, mild conditions for in vivo treatment might be insufficient to compete with the intracellular source of rafts contributed by Golgi vesicles. To circumvent this problem, we fractionated cells and isolated microsome fractions and treated them with a higher concentration of MCBD than the one normally used for in vivo studies. As shown in Fig. 3 (A–C), MCBD treatment under these conditions shifted the pattern of all of the subunits of DNA-PK toward the soluble fraction of the gradient.

Finally, the localization of DNA-PK components in lipid rafts was confirmed in at least three independent preparations of the human cell line MO57 K and J and extended to other human cells (lymphocytes and tonsil), mouse embryonic fibroblasts, and Chinese hamster ovary (CHO) cell lines.

Independent Localization of DNA-PK Subunit into Lipid Rafts—DNA-PK is a heterotrimeric protein complex composed of Ku70, Ku86, and DNA-PKcs, which are assembled at DSBs. It has been previously shown that DNA-PKcs and Ku do not
associate in the absence of DNA ends (47, 48); however, a recent report has challenged this notion (49).

In an attempt to understand the mechanism by which the DNA-PK complex is localized in lipid rafts, we prepared DIGs from human cells defective in DNA-PKcs, MO57K, and from controls MO57K (50), from CHO cell line 

$xrs-6$, which we characterized previously as carrying a mutation in the Ku86 gene (51, 52), and from its parental cell line CHO-K1. We and others demonstrated that the absence of Ku86 impairs the recruitment of DNA-PKcs to DNA ends. Thus, 

$xrs-6$ cells lack kinase activity, which in turn renders the cell line radiosensitive and defective in V(D)J recombination. In addition, the absence of Ku86 affects the stability of Ku70, and reduced levels of Ku70 are seen in 

$xrs-6$ when compared with parental control CHO-K1 (53).

As shown in Fig. 4, the absence of DNA-PKcs does not affect localization of Ku70 (A and B) or Ku86 (not shown) into lipid rafts. Similarly, mutation in Ku86 does not impact on the localization of DNA-PKcs into lipid rafts (D–E). It is worth noting that the level of the DNA-PK complex in rodents is nearly 20-fold less than in humans, explaining the reduced signal in Fig. 4, D–E, even after concentration of the sample. These results show that the DNA-PK complex needs not be preassembled to be localized into DIGs and suggest an independent interaction of the subunits directly or indirectly with the plasma membrane proteins.

Finally, one of the most important properties of lipid rafts is that they can include or exclude proteins to a variable extent in response to a stimulus. This regulatory aspect of DIGs was included three membrane proteins, epidermal growth factor receptor (EGFR), calnexin, and Lyn, which, as expected, associate in the absence of DNA ends (47, 48); however, a recent report has challenged this notion (49).
DNA-PK is a membrane-associated complex. Microsomes were prepared as described under “Experimental Procedures” and treated in the absence (Cont) or presence of Na₂CO₃ (0.1 M, pH 11.5) (Alk) or KBr (1 M) (KBr). Equal amounts of protein (3 μg) for microsome fractions (pellet) and equal volumes (40 μl) of supernatant (supernat) were resolved by SDS-PAGE and detected by Western blotting with specific antibodies. A, the same membrane was first hybridized with anti-DNA-PKcs (top) and subsequently with anti-Ku86 (middle) and finally anti-EGFR (bottom). B, a new membrane was first hybridized with anti-Ku70 (top) and subsequently with anti-calnexin (middle) and finally with anti-Lyn (bottom).

Fig. 5. DNA-PK is a membrane-associated complex. Microsomes were prepared as described under “Experimental Procedures” and treated in the absence (Cont) or presence of Na₂CO₃ (0.1 M, pH 11.5) (Alk) or KBr (1 M) (KBr). Equal amounts of protein (3 μg) for microsome fractions (pellet) and equal volumes (40 μl) of supernatant (supernat) were resolved by SDS-PAGE and detected by Western blotting with specific antibodies. A, the same membrane was first hybridized with anti-DNA-PKcs (top) and subsequently with anti-Ku86 (middle) and finally anti-EGFR (bottom). B, a new membrane was first hybridized with anti-Ku70 (top) and subsequently with anti-calnexin (middle) and finally with anti-Lyn (bottom).

DNA-PK complex might have in the plasma membrane, we compared the protein phosphorylation pattern of lipid rafts isolated from a human cell line defective in DNA-PKcs (MO57J) and control (MO57K) after irradiation. This was achieved by in vivo labeling with [³²P]orthophosphate and lipid rafts isolated and separated by two-dimensional PAGE. Signals are detected by autoradiography, and differences are shown in Fig. 7A (see arrows). To control for equivalent protein loading, silver-stained pattern of two-dimensional gels showed equivalent protein loading (see Fig. 7B).

We interpreted these results as an activation of DNA-PK directly or indirectly in response to IR treatment that leads to a phosphorylation of raft proteins. This, in turn, leads us to speculate that a novel role that this complex may have is in signal transduction of the IR response.

Experiments are in progress to identify these substrates present in lipid rafts by mass spectroscopy. In the same context, a differential phosphorylation pattern has been observed from rafts isolated from the CHO cell line V-3, defective in DNA-PK activity, when compared with its parental control AA8 (data not shown).

**DISCUSSION**

Increased sensitivity to IR has been shown to be due to defects in DSBR and mutations in the proteins that detect DNA damage. However, it is now recognized that the cellular radiation response is complex, and radiosensitivity may be regulated at different levels.

Radiation also induced rapid activation of acid sphingomyelinase rapid sphingomyelin, hydrolysis, and ceramide production in a nuclear free cell lysate, indicating a direct effect of radiation on cytoplasmic membrane, independent of the effect on nuclei. In addition, sphingomyelinase activities have also been observed in sphingomyelin-rich plasma membrane microdomains, caveolae, which are a specialized subset of lipid rafts (for reviews, see Refs. 38 and 56). A direct relationship...
between resistance to radiation-induced apoptosis and defective ceramide signaling has been established.

Furthermore, exposure to IR triggers intracellular signaling cascades that overlap with pathways initiated by ligand engagement to a receptor (23–32), and a variety of kinases including PKC and Raf-1 kinase are activated after IR treatment (33-37).

In addition, a common product of the exposure to IR is the generation of reactive oxygen species, which in turn generates hydrogen peroxide (H$_2$O$_2$). Many receptors (EGFR, B cell receptor, and insulin receptor kinase to name a few) start to signal in a ligand-independent manner when cells are treated with either H$_2$O$_2$ or even stronger oxidants. This indicates that H$_2$O$_2$ can mimic the ligand engagement process, and a list of possible alternatives to explain this activation phenomenon may include direct oxidation of the receptor that leads to aggregation, cross-linking, or conformational changes. Alternatively, H$_2$O$_2$ could activate intracellular protein kinases involved in the signal transduction pathway associated with this receptor. A third possibility, not mutually exclusive, involves the inactivation of protein phosphatases by H$_2$O$_2$ (for a review, see Ref. 57).

It is universally recognized that lipid rafts play a crucial role in the initiation and organization of signaling cascades, since they spatially concentrate or exclude components of the signaling machinery. Of note, another protein involved in the response to IR, ATM, has also been reported to localize outside the nucleus in membrane-associated vesicles. Furthermore, lymphoblast cells isolated from patients carrying a mutation in the ATM gene were defective in the IR-induced activation of a raft-resident protein-tyrosine kinase p53/56 Lyn (44).

To unravel the potential role of the DNA-PKcs in signal transduction, we searched for its localization in lipid rafts (shown in Figs. 1–3). Results from this study show that the holoenzyme DNA-PK, but not another member of the NHEJ machinery, XRCC4, is localized in these membrane-associated compartments. In addition, this localization in DIGs is not a general characteristic of proteins associated with DNA repair protein complexes, because representatives of other DNA repair pathways such as MSH2 (45), MSH6 (data not shown), or BLM-1 (data not shown) do not localize to lipid rafts. This in turn suggests that DNA-PK may be involved in the response to IR independent of its role in DSBR. These results warrant further experiments to determine the degree to which DNA-PK activity is dependent on DNA damage.

One of the most important properties of lipid rafts is that they can variably include or exclude proteins in response to a stimulus. However, as shown in Fig. 4 (A–E), relocation of any of the subunits of the DNA-PK was not dependent on irradiation.

Although DNA-PKcs is absent in yeast, functional homologues of Ku70 and Ku86 exist. Results from this study show the localization of Ku86 in lipid rafts isolated from yeast. Lipid rafts in yeast are similar to mammalian rafts but contain ergosterol instead of cholesterol (see Fig. 6).
In an attempt to understand the physiological role that the presence of DNA-PK complex might have in the plasma membrane, we compared the protein phosphorylation pattern of lipid rafts isolated from a human cell line defective in DNA-PKcs, M057J, and a control, M057K, after irradiation. This was achieved by in vivo labeling with [32P]orthophosphate and subsequent separation of lipid raft fractions using two-dimensional PAGE. Clear differences can be seen in Fig. 7A. These phosphorylation changes appear to be mediated directly or indirectly by DNA-PK activation. Efforts are under way to identify by mass spectroscopy putative targets.

At present, the mechanism of activation of DNA-PK in lipid rafts has not been determined, and a signal from DNA damage may be involved. In fact, this alternative activation pathway might be Ku-dependent as well. In response to IR, an allosteric modification in the DNA-PKcs subunit may lead to a direct activation of its kinase activity in the absence of DNA and/or to an alteration of its ability to interact with other proteins present in DIGs. Although DNA-PK is known to be activated by DNA ends, recent compelling evidence suggests that its kinase activity can also be stimulated by interacting proteins such as thyroid hormone receptor-binding protein or even certain Ab proteins. In this study, we demonstrated to be an allosteric function of C1B that allows the binding of DNA-PKcs to Ku in the absence of DSBs (61). In addition, C1B is a calcium binding protein that is N-terminally myristoylated and has been identified to bind to two plasma membrane proteins: integrin α1β2 (72) and preselinlin 2 (73). Interestingly, the C-terminal portion of either mouse or human C1B harbors a consensus signature of proteins that interact with PDZ domains. In addition, an x-ray-induced Ku70-binding protein associated with cell death, clusterin/XIP8/apolipoprotein J, is known to be present in plasma membrane and is involved in membrane lipid recycling, apoptosis, and other functions (74). Although the original report associated Ku70 with only the nuclear form of clusterin, the membrane-associated isoform may be present at low concentrations undetectable without an enrichment of plasma membrane proteins.

It is worth noting that a recent report provides evidence for the role of DNA-PK in innate immune response. In this study, in vitro activation of bone marrow-derived macrophages with bacterial DNA isolated from DNA-PKcs −/− mice was impaired in interleukin-6 and -12 production, and this pathway is dependent on activation of IKK and NF-κB (75). The authors speculated about the possibility of a DNA-PK-dependent signaling cascade, but its internal (nuclear/cyttoplasmic) localization does not support this hypothesis. Thus, the localization of DNA-PKcs in lipid rafts reported in this study might help to reconcile this conundrum. Additionally, NF-κB has been demonstrated to be an in vitro substrate for DNA-PK and also to be involved in IR response (76). Consistent with their role in signal transduction, we and others (77) localized these factors in DIGs.

Equally intriguing is the involvement of membrane signaling

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8 H. Lucero, D. Gae, G. E. Taccioli, unpublished data.
DNA-PK in Lipid Rafts

in the bystander effect in irradiated cells and its impairment by destabilization of lipid raft after cholesterol sequestration (78). Experiments are under way to address the possibility that DNA-PK might be involved in this process.

In closing, we have provided compelling evidence that shows a novel localization of the DNA-PK complex in lipid rafts and evidence that this recruitment is not a generalized property of factors associated with the NHEJ machinery. This localization is probably an interaction with resident membrane proteins. Results from this study provide provocative evidence for the existence of an additional role of the DNA-PKcs-dependent plasma membrane phenomena, which do not reconcile with the known response to IR at the membrane level and its dependence on DNA damage. These results may shed some light on understanding the mechanism of some DNA-PKcs-dependent plasma membrane phenomena, which do not reconcile with the known nuclear and cytoplasmic localization of this protein complex.

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Additions and Corrections

Vol. 278 (2003) 22136–22143

Novel localization of the DNA-PK complex in lipid rafts. A putative role in the signal transduction pathway of the ionizing radiation response.

Hector Lucero, Darren Gae, and Guillermo E. Taccioli

Page 22136: The following grant statement should be included: “Dr. Lucero is supported by National Institutes of Health Grant GM31318.”

Vol. 278 (2003) 32005–32013

Age-related changes in the biomolecular mechanisms of calvarial osteoblast biology affect fibroblast growth factor-2 signaling and osteogenesis.

Catherine M. Cowan, Natalina Quarto, Stephen M. Warren, Ali Salim, and Michael T. Longaker

The word “calvarial” was misspelled. The corrected title is shown above.

Vol. 278 (2003) 36959–36965

CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation.

Krisztian A. Kovacs, Myriam Steinmann, Pierre J. Magistretti, Olivier Halfon, and Jean-Rene Cardinaux

Page 36965, line 3: The sentence should read, “Similarly, the cyclin E-Cdk2 complex, which may target the same sites as MAPK, phosphorylates CBP in a cell cycle-dependent manner, thus enhancing its HAT activity (71).”

Vol. 278 (2003) 40425–40428

YXXM motifs in the PDGF-β receptor serve dual roles as phosphoinositide 3-kinase binding motifs and tyrosine-based endocytic sorting signals.

Haiyan Wu, David A. Windmiller, Ling Wang, and Jonathan M. Backer

The PDGFR-F/F mutant was not a new construct but rather was a gift from Dr. Jonathan A. Cooper, Fred Hutchinson Cancer Research Center, Seattle, WA. The authors would like to thank Dr. Cooper and regret the omission of this acknowledgment.

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