Defective regulation of Ca^{2+}/calmodulin-dependent protein kinase II in γ-irradiated ataxia telangiectasia fibroblasts

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Abstract Recent indirect evidence suggests that a Ca^{2+}/calmodulin-dependent pathway, which may involve calmodulin-dependent protein kinase II (CaMKII), mediates the S-phase delay manifested by γ-ray-exposed human fibroblasts. This pathway is severely impaired in ataxia telangiectasia (A-T) cells [Mirzayans et al. (1995) Oncogene 11, 1597]. To extend these findings, we assayed CaMKII activity in irradiated normal and A-T fibroblasts. The radiation treatment induced the autonomous activity of the kinase in normal cells. In contrast, this activity was not elevated in either (i) normal cells pretreated with the selective CaMKII antagonist KN-62 or (ii) γ-irradiated A-T cells. Moreover, A-T fibroblasts, unlike normal cells, failed to mobilize intracellular Ca^{2+} upon mitogenic stimulation. These findings identify a novel role for CaMKII in radiation-induced signal transduction and suggest its involvement in effecting the S-phase delay. The data also implicate ATM, the product of the gene responsible for A-T, as a key mediator of both intracellular Ca^{2+} mobilization and CaMKII activation in response not only to genotoxic stress but also to physiological stimuli.

Key words: Ataxia telangiectasia; Calmodulin-dependent protein kinase II; DNA synthesis; Intracellular Ca^{2+} mobilization

1. Introduction

Many protein kinases are activated by ionizing radiation, both serine/threonine kinases (protein kinase C (PKC), $pp90^{srk}$, MAP kinases and SAP/JNK kinases) and tyrosine kinases ($p56/53^{lyn}$ and c-Abl) (for review, see [1]). Activation of kinases seems to be responsible for the radiation-induced perturbations in cell-cycle progression. For example, radiation-induced G_1 arrest is thought to be orchestrated by PKC [2] and c-Abl activation [3]. G_2 arrest, on the other hand, can be linked to phosphorylation of $p34^{cdc-2}$ kinase [4] or activation of the novel protein kinase Chk1 [5]. Little information is available, however, on the precise mechanism governing the S-phase delay caused by ionizing radiation. We have recently reported that a Ca^{2+}/calmodulin-dependent pathway, which may involve calmodulin-dependent protein kinase II (CaMKII), mediates this delay. Our data also demonstrate that this pathway is malfunctional in ataxia telangiectasia (A-T) fibroblasts and may account for the typical lack of S-phase delay experienced by these cells on exposure to γ-rays [6]. Indeed, the cellular phenotype of A-T points to defects in the signal transduction pathways that mediate cell-cycle checkpoint controls, as well as differentiation and development [7]. In this report we show that exposure of normal human fibroblasts to ionizing radiation elicits rapid activation of CaMKII. However, this activation is severely compromised in A-T fibroblasts. Moreover, A-T fibroblasts exhibit a defect in the mobilization of intracellular Ca^{2+}. These observations lead us to propose that CaMKII is intimately involved in radiation-induced signal transduction. In addition, the product of *ATM*, the gene mutated in A-T, appears to be regulating CaMKII activity indirectly by enabling cells to respond to external stimuli through release of intracellular Ca^{2+}.

2. Materials and methods

2.1. Cell cultivation and γ-ray treatment

Human fibroblast strains, both normal (GM38) and A-T (AT2BE), were cultivated as described elsewhere [6]. Experimental cell cultures were first incubated in serum-free medium for 90 min. Where indicated, KN-62 (10 μM) was added to the medium 30 min before cell irradiation. Exposure to ^{60}Co γ-rays (dose rate ∼60 Gy/min) was carried out at room temperature as detailed earlier [6]. Unless indicated otherwise, within 15–55 s after irradiation, cells were washed in ice-cold PBS, placed on ice and scraped into the lysis buffer (200 mM NaCl, 40 mM Tris-HCl pH 7.5, 2 mM 2-mercaptoethanol, 0.5 mM EDTA and EGTA, 10 mM tetrasodium pyrophosphate, 0.4 mM sodium molybdate, 20 mM β-glycerophosphate, 1% Nonidet P-40, 10 μg/ml of leupeptin and 0.1 mM PMSF). Lysates were homogenized in a Dounce homogenizer and cleared by centrifugation.

2.2. CaMKII activity assays

Measurements were done according to [8]. Autonomous kinase activity was determined in the presence of 2 mM EGTA and 20 μM autocamtide-2 (Biomol Research Laboratories, Inc., Plymouth, PA, USA). Calmodulin-dependent (total) activity was measured in the presence of 0.5 mM $CaCl_2$ and 5 μg/ml of calmodulin (Sigma), while EGTA was omitted. Reactions, run in triplicate, were initialized by adding 3–5 μg of total extracted protein, carried out for 2 min at 37°C, and terminated by adding ice-cold TCA (5% final concentration). The kinase activity was expressed as pmol (or nmol) of ^{32}P incorporated into the peptide substrate by 1 mg of total extracted protein during 1 min. Zymography and calmodulin binding assays were done according to [9] and [10], respectively.

2.3. Reverse transcriptase-coupled polymerase chain reaction (RT-PCR)

mRNA was purified from normal and A-T fibroblasts according to the Oligotex Direct mRNA protocol (Qiagen Inc., Valencia, CA, USA). To define the mRNA product(s), nested primers, whose sequences corresponded to human CaMKII γ cDNA, were used: nucleotides 904–926 and 1060–1081 [11,12]. PCR products were then cloned into pCR 2.1 (Invitrogen, San Diego, CA, USA) and the inserts were sequenced directly using a cyclic sequencing kit (#402079) from Applied Biosystems (Foster City, CA, USA). Electrophoresis and sequence analysis were conducted on an automatic sequencing apparatus (Applied Biosystems 373A).

2.4. Intracellular Ca^{2+} mobilization

Changes in intracellular Ca^{2+} levels were measured by confocal microscopy (Leica, Heidelberg, Germany) using the fluorescent Ca^{2+}

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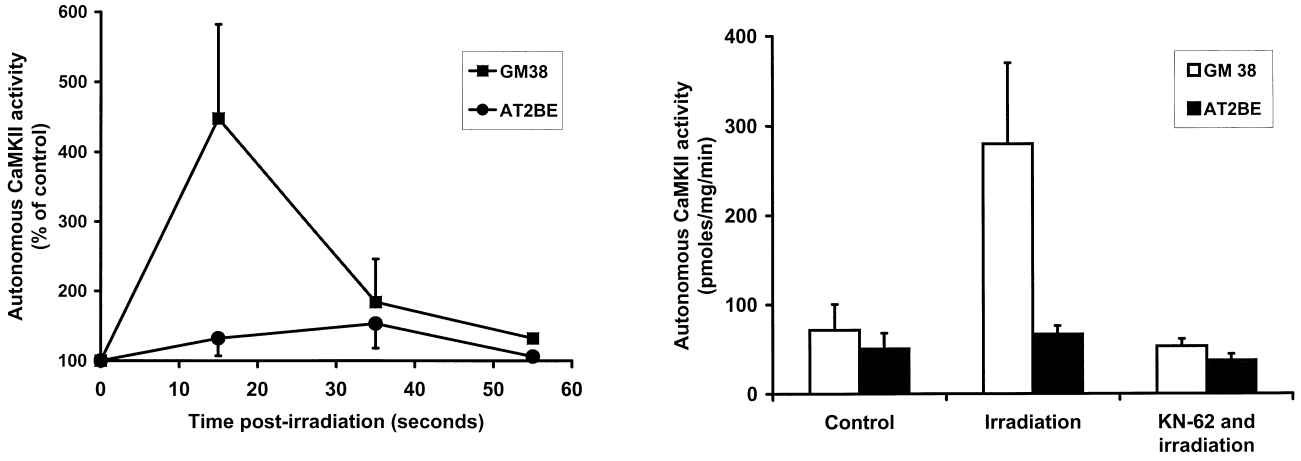

Fig. 1. Autonomous activity of CaMKII in γ-ray-treated human (normal and A-T) fibroblasts. Left panel: time course of kinase activation. Mean values ± S.D. of three experiments are shown. Right panel: effect of KN-62. Kinase activity was measured in cell extracts obtained 15 s after irradiation. Mean values ± S.D. of six experiments are shown.

indicator fluo-3 AM (Molecular Probes, Eugene, OR, USA). Cells (4×10^5 per 100 mm dish) were cultured on glass coverslips for 2 days, serum-starved for 12 h, and loaded with 1 μM indicator in fresh RPMI 1640 medium for 30 min at 37°C. The coverslips were then transferred to prewarmed medium and Ca^{2+} mobilization was triggered by adding fetal calf serum (10% final concentration).

3. Results

3.1. Gamma-ray-mediated induction of the autonomous CaMKII activity occurs in normal fibroblasts but is delayed and attenuated in A-T fibroblasts

The principal aim of this investigation was to explore the involvement of CaMKII in the response of human fibroblasts to ionizing radiation. Normal (GM38) and A-T (AT2BE) fibroblast cultures were irradiated with 10 Gy, incubated for indicated times, and lysed. Total cell extracts were then tested for their ability to phosphorylate autocamtide-2, a specific CaMKII substrate representing the kinase's autophosphorylation sequence, in the absence of Ca^{2+} and calmodulin. In normal fibroblasts ionizing radiation stimulated the autonomous kinase activity 4.5-fold within 15 s (Fig. 1, left panel).

In AT2BE fibroblasts, however, stimulation of the kinase activity was delayed and reached only 1.5-fold above basal level after 35 s. The observed up-regulation of kinase activity in normal fibroblasts presumably reflects its in vivo activation caused by the increase in intracellular Ca^{2+} and calmodulin binding [13]. To validate this assumption, we pretreated normal cells with KN-62, a selective CaMKII inhibitor. KN-62 inhibits the kinase by competing with the Ca^{2+}-induced binding of calmodulin to the enzyme [14], thus preventing its activation. Preincubation of GM38 fibroblasts with KN-62 prevented radiation-induced kinase activation (Fig. 1, right panel). Induction of CaMKII autonomous activity can also be alleviated by anti-calmodulin compounds W7 and W13. These inhibitors, like KN-62, confer a delay in S-phase progression in irradiated normal cells [6]. Hence, we conclude that γ-ray treatment proficiently induces Ca^{2+}/calmodulin-dependent formation of autonomous CaMKII in normal fibroblasts, whereas A-T fibroblasts are severely compromised. The disparate effect of radiation treatment was not unique to the two cell strains shown here, since similar differences in response were observed in GM43 (normal) compared to AT3BI or AT5BI (A-T) fibroblast strains. Yet CaMKII could be stimulated to the same extent in both normal and A-T fibroblasts (0.8 nmol of incorporated ^{32}P/mg/min) by exposing the cells to ionomycin, a calcium ionophore, for 20 s. We also carried out the phosphorylation reaction under in vitro conditions, in which both Ca^{2+} and calmodulin were present so that all CaMKII molecules could undergo maximal stimulation. The total kinase activity measured in unirradiated normal fibroblasts was 8.2 ± 1.5 nmol/mg/min of incorporated ^{32}P and 8.6 ± 1.6 in cells exposed to radiation. A-T fibroblasts exhibited even slightly higher total kinase activity, yielding 12.8 ± 2.0 nmol/mg/min of incorporated ^{32}P in untreated cells and 12.1 ± 1.9 in irradiated cells. These data show that A-T fibroblasts possess fully functional CaMKII and its intracellular level is comparable to that present in normal fibroblasts.

3.2. Detection of CaMKII by zymography, calmodulin binding and RT-PCR

Zymography is a useful procedure for identifying the apparent molecular weight of a given kinase [9]. We employed this technique to confirm that exposure of normal human fibroblasts to γ-rays converts CaMKII to its autonomous form. Extracts of irradiated normal fibroblasts showed the presence of a 55 kDa band, the intensity (radioactivity) of which was demonstrably stronger than that present in the unirradiated control (Fig. 2, left panel). In contrast, the intensities of the other two prominent bands, corresponding to 47 kDa (lower band) and 83 kDa (upper band) polypeptides, respectively, were similar in samples obtained from control and irradiated fibroblasts.

The upper band may represent an unknown autophosphorylating protein, whereas the lower band may be a degradation product of CaMKII. Moreover, only the 55 kDa polypeptide was capable of Ca^{2+}/calmodulin-dependent autophosphorylation (not shown). Hence, we conclude that the 55 kDa polypeptide represents the autonomous form of CaMKII. No difference was noted in the intensities of the 47, 55 and 83 kDa bands between untreated and γ-treated A-T cells (not shown). If the 55 kDa polypeptide represents a CaMKII monomer it should bind the Ca^{2+}/calmodulin complex. To test this prediction, we incubated Western blots of fibroblast extracts with biotinylated calmodulin in the presence of Ca^{2+}. Fig. 2 (right

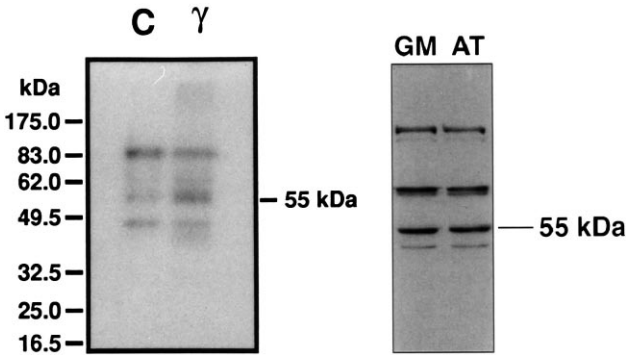

Fig. 2. Zymography (left panel) and calmodulin overlay (right panel) of human fibroblast extracts. Zymography was done using extracts isolated from control (C) or irradiated (γ) normal (GM38) fibroblasts. 20 μM autocamtide-2 was used as a substrate and the reaction was carried out in the presence of 2.0 mM EGTA. Calmodulin overlay was done using extracts isolated from GM38 (GM) and AT2BE (AT) fibroblast cultures. No non-specific binding of calmodulin was observed in the presence of EGTA.

panel) shows a prominent 55 kDa band present in cell extracts obtained from normal and A-T fibroblasts. Its intensity was very similar in all extracts tested. Thus, the calmodulin binding analysis and zymography allowed us to demonstrate that the 55 kDa polypeptide is indeed monomeric CaMKII. Others [11,12,15] have reported that the two most common CaMKII izoenzymes, outside the brain, are CaMKII γB and γC. These proteins, which are present, for example, in rodent fibroblasts [12], migrate as 55 kDa polypeptides on SDS-PAGE gels. In order to establish if human dermal fibroblasts express these specific izoenzymes, we conducted RT-PCR analysis of mRNA isolated from normal and A-T fibroblasts. Primers used for the nested PCR were designed to amplify sequences in the variable domain of CaMKII γ, thus permitting isoenzyme distinction. The amplified products were then cloned into the pCR 2.1 vector and sequenced in both directions. The resulting sequences were identical to human CaMKII γB and γC isoforms present in human T-cells [11] and, as predicted, were 176 bp and 107 bp, respectively. No difference in the quantity of products was found, when mRNA from either normal or A-T fibroblasts was used for analysis.

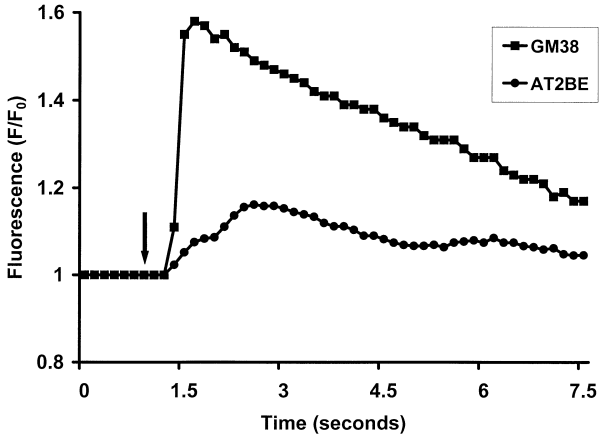

Fig. 3. Serum-induced intracellular Ca^{2+} mobilization in human fibroblasts. The arrow indicates the time at which serum was introduced. A total of 10 GM38 and 10 AT2BE cells were individually analyzed and the fluorescence values were then averaged. Three independent experiments yielded similar results.

3.3. Serum-induced intracellular Ca^{2+} mobilization

The inability of radiation-treated A-T fibroblasts to convert CaMKII into its autonomous form suggests that these cells are unable to mobilize intracellular Ca^{2+} properly irrespective of the stimulus. If this is indeed true then A-T fibroblasts should also show defective calcium ion mobilization in response to a physiological impulse. To test this notion, the Ca^{2+} transient was induced with fetal calf serum and this induction was measured using the conventional intracellular Ca^{2+} indicator fluo-3. As can be seen in Fig. 3, normal fibroblasts responded to serum stimulation with a very rapid mobilization of free Ca^{2+}, as indicated by the sharp increase in fluo-3 fluorescence. Maximal fluorescence was observed within 2 s after serum addition. A-T fibroblasts, on the other hand, responded to serum with a Ca^{2+} transient that was both diminished and protracted.

Decreased calcium storage in the endoplasmic reticulum of A-T cells was not responsible for their attenuated response to serum-residing growth factors, since a substantial increase in fluo-3 fluorescence was seen after addition of thapsigargin (not shown). We also tested the response of CaMKII to serum-induced intracellular calcium mobilization. The serum-induced increase in autonomous CaMKII activity was higher in normal fibroblasts than in A-T cells (not shown). These combined findings clearly document that A-T fibroblasts are deficient in intracellular Ca^{2+} mobilization and subsequent CaMKII activation.

4. Discussion

Irradiation of human skin fibroblasts leads to the formation of autonomous CaMKII in vivo. This conversion occurs within 15 s post-irradiation. It is well-known that the autophosphorylation of CaMKII and the ensuing autonomous activity state of the multiheteromeric protein occurs as early as 20 s after the introduction of growth factors or hormones, which are known to elicit a rapid increase in intracellular Ca^{2+} levels ([16,17], this paper). CaMKII is not, however, the sole Ca^{2+}-dependent protein kinase that is activated in vivo by ionizing radiation. PKC is also activated rapidly by ionizing radiation and intracellular chelation of Ca^{2+} attenuates this up-regulation [18]. Ionizing radiation also stimulates, albeit indirectly, mitogen-activated protein kinase (MAPK). An intracellular Ca^{2+} chelator, a Ca^{2+} antagonist or a phospholipase C inhibitor each inhibits radiation-induced Ca^{2+} oscillations and MAPK stimulation [19]. The most important findings presented in this study are that: (i) CaMKII is involved in effecting the radiation-induced signal transduction circuitry and (ii) A-T fibroblasts are deficient in this cascade. The attenuated conversion to the autonomous form, rather than decreased enzyme levels or different patterns of kinase expression, is responsible for the lack of CaMKII activation in irradiated A-T fibroblasts. Yet CaMKII could be stimulated to the same extent in both normal and A-T fibroblasts by exposing the cells to a calcium ionophore, ionomycin. These observations, and data showed in Fig. 3, indicate that A-T fibroblasts are defective in their ability to effect Ca^{2+} mobilization. Recent observations [20] also support a role for the ATM protein in the regulation of intracellular Ca^{2+}. We propose that ATM

functions as a key mediator of both intracellular Ca^{2+} mobilization and CaMKII activation in response not only to genotoxic stress but also to certain physiologic stimuli. Direct activation of c-Abl and p53 by ATM serves to effect radiation-induced G_1 arrest [3,21]. What role then might CaMKII play in the ATM-dependent delay of S-phase progression? A recent report [22] provides compelling evidence that CaMKII phosphorylates replication factor C (RF-C), thereby preventing proliferating cell nuclear antigen (PCNA) from binding to RF-C. This, in turn, leads to decreased DNA synthesis in vitro. We therefore hypothesize that the rapid activation of CaMKII in vivo may cause the phosphorylation of RF-C. As a result, PCNA may not bind to RF-C and hence the DNA replication initiation machinery stalls. Replication complexes that already contain PCNA would be, however, unaffected and drive residual DNA synthesis. Failure to activate CaMKII in response to γ-rays, caused by either insufficient Ca^{2+} mobilization or the presence of the kinase inhibitor KN-62, would attenuate RF-C phosphorylation and thereby contribute to radioresistant DNA synthesis, as is indeed observed in γ-irradiated A-T fibroblasts.

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