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Purification and identification of a protein kinase activity modulated by ionizing radiation

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Abstract

In order to identify novel protein kinases, which are involved in signal transduction processes after exposure of cells to ionizing radiation we screened HL-60 cells using an in-gel renaturation assay. Using this approach we identified a renaturable serine/threonine kinase with an apparent molecular mass of 90 kDa (pK90). The activity of pK90 dropped within minutes after exposure to a dose of 10 Gy. It reached a minimum 15–30 min after irradiation and increased back to pre-treatment values 6 h later. A down-regulation of the kinase activity was detectable after a dose of 1 Gy. Failure of H_2O_2 to down-regulate pK90 activity indicates a requirement for DNA double-strand-breaks to modulate the kinase activity. In contrast to the molecular mass of 90 kDa in SDS–PAGE we found a molecular mass of around 450 kDa for the native protein using gel filtration chromatography, indicating that pK90 forms a multi-protein complex under native conditions. To identify pK90 we partially purified the protein by three affinity chromatography steps (heparin–Sepharose, phosphate metal affinity, and Cibacron-Blue-F3G-A–Sepharose). Mass spectrometric analysis of the purified 90 kDa fraction showed that pK90 is identical to Tlk1, which was verified by immunoprecipitation.

Keywords: Protein kinase; Renaturation; Ionizing radiation; Chromatography

The cellular response to ionizing radiation and therefore the radiosensitivity of tumors and normal tissues in radiotherapy depends to a great extent on intracellular signal transduction events. Radiation induced modifications of the nuclear DNA [15] or in some cell systems of the cell membrane [11] initiate the cellular response. These initial events lead to activation of several signal transduction cascades (reviewed in [4,10]), which finally determine whether the cell will undergo radiation induced cell death by a variety of mechanisms or whether it will survive. The cellular signaling responses are diverse and include among others the activation of cell surface receptors, modulation of the activity of cytoplasmic and nuclear protein kinases, binding of transcription factors, alterations of the activity protein phosphatases, and the activation of adapter proteins. The prominent role of protein kinases like DNA-PK_{cs} [2], ATM [23], and c-Abl [27] in these cellular signal transduction processes is demonstrated by the significant alteration in the cellular radiosensitivity which can be observed if the activity of these kinases is exogenously modified.

Because of their mentioned importance and because of their attractiveness as a target for a pharmacologic modification of the cellular radiosensitivity, we screened for protein kinases, with a hitherto un-described role in the cellular response to ionizing radiation. The screening was performed in the promyelocytic leukemic cell line HL-60 using an in gel renaturation assay, initially described by Geahlen et al. [7]. In this assay cellular proteins are separated by SDS–PAGE, renaturated in the gel, and protein kinases are identified by the incorporation of ^{32}P due to in situ auto- or substrate-phosphorylation. This assay has been used successfully in the identification of numerous serine/threonine kinases like MAP kinases [8], RING3 [18] or p90rsk [14].

Using this in-gel renaturation assay [7] we identified a hitherto un-described protein kinase with an apparent molecular mass of 90 kDa , the activity of which was down-regulated after exposure to ionizing radiation. We partially purified this kinase and identified it by mass

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spectroscopy. We found that the renaturable protein kinase is identical to tousled-like kinase 1 (Tlk1), a serine/threonine kinase initially identified in plants [21]. The response of Tlk1 to ionizing radiation was recently also characterized by another group [9], verifying our identification of the renaturable kinase.

Experimental procedures

Cell culture and treatment. HL-60 cells were cultured in RPMI1640 containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified mixture of 95% air and 5% CO_2. The cells were maintained at a density lower than 5×10^5/ml. Irradiations were performed at room temperature with a RT 100 (Philips) X-ray machine at 100 kV using a 1.7 mm Al filter. The dose rate was around 13 Gy/min. For the induction of single-strand-breaks HL-60 cells were incubated for 30 min at 4 °C normal cell culture medium containing 1 mM H_2O_2. The incubation was terminated by adding 500 U/ml catalase and the cells were brought back to 37 °C.

Assays for renaturable kinases. Two different assays were used to renaturate and detect protein kinases after separation by SDS–PAGE. The first one, which renaturates and detects the kinases in situ in the gel, was essentially performed as published [7] with the only difference that 50 mM β-mercaptoethanol was used instead of DTT. The second one, which renaturates and detects the kinases in situ after transfer to PVDF membranes, was performed as described [5] with slight modifications. Again DTT was substituted by 50 mM β-mercaptoethanol and the PVDF membranes were finally washed four times for 15 min at 4 °C in 5% trichloroacetic acid containing 10 g/L tetrasodium pyrophosphate to remove the unincorporated radioactivity. For both procedures the incorporated radioactivity was measured using a Cyclone phosphoimager (Packard).

Phosphoamino acid analysis. Phosphoamino acid analysis was performed as described [3]. Briefly the band containing the pK90 signal was cut out of the PVDF membrane after the kinase assay, the proteins were hydrolyzed in 6 M HCl at 110 °C for 1 h and the radiolabelled phosphoamino acids were separated by two-dimensional thin layer electrophoresis and detected with a phosphoimager.

Subcellular fractionation. Nuclear and cytoplasmic protein fractions were prepared as described [6] with slight modifications. Briefly HL-60 cells were lysed at 4 °C in a buffer consisting of 10 mM Tris (pH 7.4), 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.3% NP-40, 0.32 M sucrose, 25 mM β-glycerophosphate, 2 mM NaF, and 1 mM PMSF. From this lysate crude nuclear and crude cytoplasmic fractions were obtained by centrifugation (900*g*, 5 min).

Alkaline phosphatase treatment. Nuclei from 5×10^5 cells were prepared as mentioned above. The nuclei were resuspended in 20 μl of the buffer used for cell lysis and the nuclear membrane was disrupted by the addition of 0.1% SDS. Thereafter 1 U of alkaline phosphatase was added and the reaction was kept at 30 °C for 15 min. The dephosphorylation reaction was terminated by the addition of 2× sample buffer and boiling. The pK90 activity was determined by the in gel kinase assay [7].

Gel filtration chromatography. From 2×10^7 HL-60 cells nuclei were isolated as described above. The nuclei were extracted in 100 μl buffer consisting of 20 mM Tris (pH 7.4), 150 mM NaCl, 50 mM β-mercaptoethanol, 5 mM $MgCl_2$, 10 mM β-glycerophosphate, and 20 mM NaF for for 10 min on ice. Insoluble particles were removed by a 16,000*g* centrifugation for 10 min. Fifty microliters of the cleared extract was injected into a Superdex 200 (Pharmacia) gel filtration column, which was run with the same buffer as used for nuclear protein extraction at a flow rate of 40 μl/min. Forty microliter fractions were collected, mixed with 40 μl of 2× sample buffer [17], and boiled for 3 min. Thirty microliters of each fraction was analyzed for pK90 activity using the renaturation assay on a PVDF membrane [5].

Partial purification of pK90. From a total of 4×10^9 HL-60 cells nuclei were isolated using the procedure described above. Nuclear proteins were extracted for 10 min on ice in a buffer consisting of 50 mM Tris (pH 7.4), 400 mM NaCl, 5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 5 mM β-glycerophosphate, 10 mM NaF, and 1 mM PMSF. A volume of 1 ml extraction buffer was used for 10^8 cells. After the removal of insoluble material by a 5 min centrifugation at 16,000*g* the extract was frozen in LN_2 and stored at −70 °C until use. After thawing 40 ml of nuclear extract was diluted with 120 ml buffer A (20 mM Tris (pH 7.2), 50 mM β-mercaptoethanol, 5 mM EDTA, and 1 mM NaF). Insoluble material was removed by centrifugation at 16,000*g* for 10 min. The extract was loaded onto a 5 ml HiTrap Heparin HP column (Pharmacia). From this column pK90 was eluted with a linear gradient ranging from 0.1 to 1 M NaCl in buffer A. Fractions were analyzed for pK90 activity using the renaturation assay on a PVDF membrane [5]. For phosphate metal affinity chromatography (PMAC) [1] the BD Phosphoprotein Enrichment Kit (Becton Dickinson) was used according to the instruction given by the manufacturer. Briefly the proteins from the 5 ml fraction containing the pK90 activity were dialyzed into the loading buffer of the BD Phosphoprotein Enrichment Kit by spin dialysis. The proteins were loaded on the column and after removal of the unbound proteins by a wash step the pK90 containing fraction was recovered in 2 ml elution buffer. For the final chromatographic step the 2 ml fraction was diluted with 8 ml buffer A and loaded onto a 1 ml HiTrap Blue HP column (Pharmacia). The column was eluted with a linear gradient ranging from 0 to 1 M KSCN in buffer A containing 100 mM NaCl. The fractions containing the pK90 activity were TCA precipitated and the proteins were separated on a 6% SDS–PAGE gel. The gel was silver stained using the protocol published in [25]. The protein band, which co-localized with the pK90 activity, was cut out of the gel and subjected to mass spectrometric analysis.

Mass spectrometry. Mass spectrometric protein identification was performed on a contract basis at the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics from the Polish Academy of Sciences, Warszawa. Briefly the proteins in the gel slice were digested with trypsin and subjected to nano-HPLC-MS/MS and the ion spectra were analyzed using the MASCOT software [19].

Immunoprecipitation. Nuclei from 3.3×10^6 HL-60 cells were prepared as mentioned above. The nuclei were lysed in RIPA buffer [22] containing 5 mM β-glycerophosphate, 10 mM NaF, and 1 mM PMSF. Insoluble proteins were removed by centrifugation (16,000*g*, 10 min) and 2.5 μg of anti-Tlk1 antibody (Zymed) was added. After an incubation for 1 h at 4 °C 20 μl of protein A–agarose was added and the incubation was continued for another hour. After three 15 min washes in RIPA buffer the samples were boiled in sample buffer and analyzed using the assay for renaturable protein kinases [5].

Results

Identification of a renaturable kinase activity

We used an in gel renaturation assay [7] to screen for protein kinases in HL-60 cells, which show a modulation of their activity after exposing the cells to ionizing radiation. With this screening assay we identified a band in the autoradiogram with an apparent molecular mass of about 90 kDa, which exhibited a lower activity in samples from irradiated cells. As shown in Fig. 1A the activity of this band dropped significantly within 5 min after irradiation of HL-60 cells with a dose of 10 Gy. Fifteen to thirty minutes after irradiation a minimum activity could be observed. At later time-points the intensity of the band increased again and reached control

values 6 h after irradiation. To investigate the dose dependence of the observed down-regulation of the 90 kDa band, HL-60 cells were irradiated with doses increasing from 1 to 20 Gy and assayed for the renaturable kinase activity 30 min later. Fig. 1B shows that a detectable down-regulation of the intensity of the 90 kDa band could be observed after doses as low as 1 Gy. At higher doses the down-regulation gets more pronounced in a dose-dependent manner and the activity reaches a plateau at doses of 10 Gy or higher.

The renaturable activity can be attributed to a protein kinase

To test whether the observed autoradiographic band at about 90 kDa is indeed caused by the covalent phosphorylation of amino acid by a renaturable protein kinase, a phosphor-amino acid analysis was performed. Fig. 2 shows the result of the two-dimensional

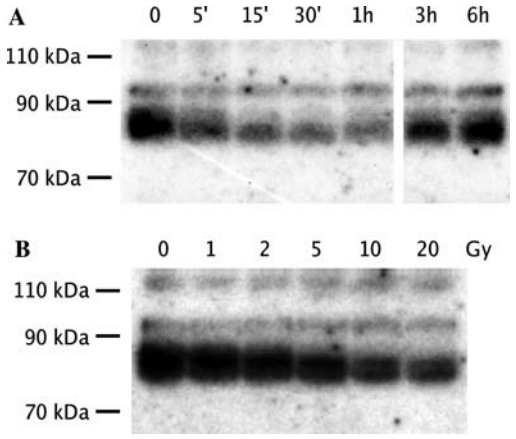

Fig. 1. Autoradiogram of an in-gel renaturation assay showing the activity of a renaturable protein kinase (pK90) slightly below the 90 kDa marker. (A) Modulation of the kinase activity at various time points after irradiation of HL-60 cells with a dose of 10 Gy. Total cellular proteins from 5×10^5 cells were loaded in each lane. (B) Dose–response of the kinase activity 30 min after irradiation with the doses mentioned. Again total proteins of 5×10^5 cells were loaded.

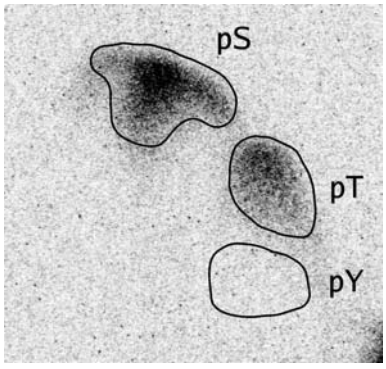

Fig. 2. Phosphoamino acid analysis of the proteins phosphorylated by pK90 in an in-gel renaturation assay. Phosphoamino acids were obtained by protein hydrolysis and separated by 2D electrophoresis. The marked areas on the autoradiogram correspond to the spots of the non-radioactive phosphoamino acids used as markers.

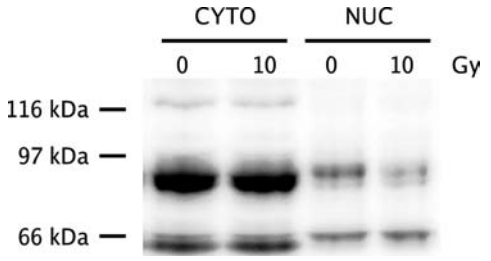

Fig. 3. Subcellular distribution of the pK90 activity. Cytoplasmic and nuclear proteins from 7.5×10^5 HL-60 cells were analyzed by an in situ renaturation assay on a PVDF membrane. Only the nuclear fraction shows an activity around 90 kDa, which is down-regulated 15 min after irradiation with a dose of 10 Gy.

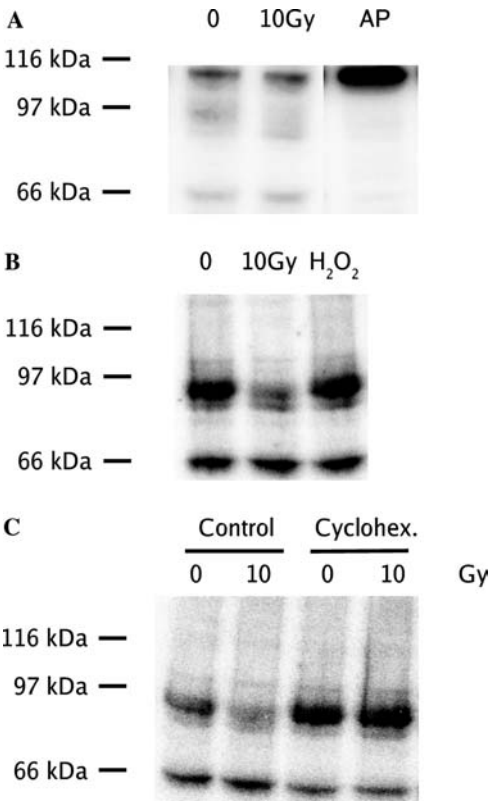

Fig. 4. (A) In situ kinase assay on a PVDF membrane showing the effect of alkaline phosphatase (AP) treatment of nuclear proteins. Nuclear proteins from 7.5×10^5 HL-60 cells were loaded in each lane. The right lane with the AP treated proteins shows that de-phosphorylation leads to a complete loss of the pK90 signal in the autoradiogram. (B) In situ renaturation assay showing that radiation induced double-strand-breaks but not H_2O_2 induced single-strand-breaks lead to a down-regulation of the pK90 activity. H_2O_2 was used at a concentration of 1 mM for 30 min on ice. The time after irradiation and peroxide treatment was 15 min. Assay condition and cell numbers were the same as in (A). (C) Pre-treatment of HL-60 cells with 35 μM cycloheximide for 1 h leads to an increase in pK90 activity and it inhibits the radiation induced down-regulation of the kinase activity. Cell numbers and assay condition are equal to the ones mentioned for (A).

electrophoretic separation of the phosphoamino acids found in the 90 kDa band. Radiolabelled phosphoserine and phosphothreonine but no phosphotyrosine were found. Therefore it can be concluded that the observed 90 kDa band results from the activity of serine/threonine kinase which we named pK90. Separation of the radiolabelled proteins in the 90 kDa band by isoelectric focusing yielded numerous bands in the autoradiogram (data not shown). This indicates that renaturated pK90 phosphorylates numerous proteins, which are found close to the position of the kinase in the gel.

Subcellular localization of pK90

For the determination of the subcellular localization of pK90, HL-60 cells were separated into a crude cytoplasmic and nuclear fraction. Both protein fractions were separated by SDS–PAGE, transferred to a PVDF membrane, and assayed for pK90 activity [5]. It can be seen in Fig. 3 that the cytoplasmic fraction of HL-60 cell did not contain any renaturable kinase activity responsive to ionizing radiation. In contrast an activity could be detected in the nuclear fraction, which was down-regulated after irradiation, indicating that pK90 is a nuclear kinase.

pK90 activity is regulated by phosphorylation

As the activity of numerous cellular kinases is regulated by phosphorylation we tested whether this is also true for pK90. As shown in Fig. 4A treatment of nuclear proteins with alkaline phosphatase led to a complete loss of the pK90 activity in the renaturation assay, thus indicating that the activity of pK90 is regulated by phosphorylation. The kinetics of ^{32}P incubation was linear in the renaturation assay on PVDF membranes [5] for the first hour (data not shown), indicating that auto-phosphorylation did not activate pK90.

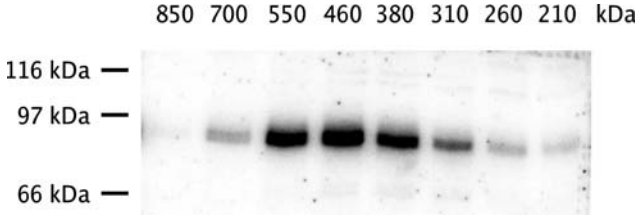

Fig. 5. In situ renaturation assay on a PVDF membrane of the fractions from a gel-filtration column. The molecular masses which are given on top of the lane were obtained by column calibration. No pK90 activity was found at its monomeric molecular mass of 90 kDa.

pK90 inactivation requires DNA DSBs and protein synthesis

To determine which type of radiation induced DNA damage is responsible for the down-regulation of pK90 activity we compared the effect of ionizing radiation to the effect of hydrogen peroxide. The autoradiogram shown in Fig. 4B demonstrates that pK90 activity decreases after irradiation of cells with a dose of 10 Gy but not after treating the the cells with 1 mM H_2O_2. As it has been demonstrated [13] that radiation induces far more double-strand-breaks for the same amount of single-strand-breaks than H_2O_2 it can be concluded that DNA double-strand-breaks are the relevant lesion for the modulation of pK90 activity. To test whether protein synthesis is required for the radiation induced down-regulation of pK90, HL-60 cells were treated with cycloheximide for 1 h prior to irradiation. It can be seen in Fig. 4C that cycloheximide inhibited the radiation induced drop in pK90 activity.

Native molecular mass of pK90

To determine the native molecular mass of pK90 an extract from HL-60 nuclei was separated on a calibrated gel-filtration column. The amount of pK90 activity found in the various fractions is shown in Fig. 5. The main peak of activity elutes at a size of around 460 kDa, indicating that pK90 exists as a multi-protein complex in its native form.

Partial purification of pK90

To purify pK90, a nuclear protein extract was prepared and sequentially purified by three affinity chromatography steps. A summary of the purification procedure is given in Table 1. Fig. 6A shows the result of the heparin affinity chromatography and Fig. 6B the one of the dye affinity step. It is obvious from Table 1 that the PMAC step did not lead to an increase in the overall purification of pK90. This step was however necessary to remove contaminating proteins from the 90 kDa region, as an omission of this step resulted in an inability

Table 1
Summary of purification protocol for pK90

Step	Total protein	Activity/μg	Activity	Yield (%)	Purification
HL-60 nuclei	320 mg	3.6	1.1×10^6	100	1
Extract	19.2 mg	59	1.1×10^6	96	16
Heparin HP	3.2 mg	159	5.1×10^5	44	44
PMAC	368 μg	134	4.9×10^4	4.3	37
Blue HP	10 μg	753	7.5×10^3	0.7	209

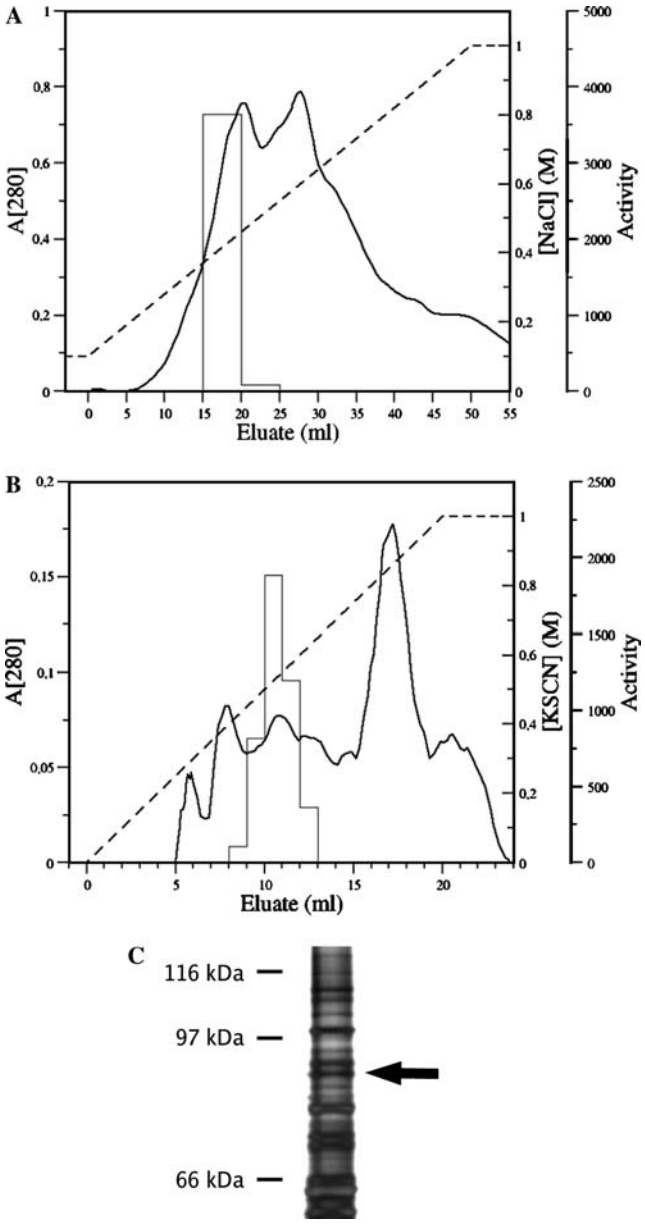

Fig. 6. Partial purification of pK90 by affinity chromatography. (A) Chromatogram of the initial heparin affinity chromatography step. The column was eluted with a linear gradient ranging from 0.1 to 1 M NaCl. The activities mentioned are the integrated digital light units measured by the phosphoimager after renaturation of the fractions on a PVDF membrane. (B) Chromatogram of the final affinity step using a Cibacron Blue F3G-A dye-affinity column. The column was eluted with a linear gradient ranging from 0 to 1 M KSCN. The activities were determined as mentioned above. (C) Silver stained gel showing the proteins present after the last purification step. The protein band marked by the arrow co-localized with the pK90 activity in the renaturation assay and was therefore excised and analyzed by nano-HPLC-MS/MS.

of the mass spectroscopy to identify pK90. The protein bands, which were detectable in a silver stained SDS–PAGE gel, are shown in Fig. 6C. The band, which is marked by an arrow in Fig. 6C, co-localized with the renaturable kinase activity and was therefore excised and subjected to mass spectroscopy.

Mass spectrometric identification of pK90

The result of the nano-HPLC-MS/MS analysis of the excised gel fragment is shown in Table 2. After the removal of signals originating from contaminating keratins three proteins could be identified in the sample. Among those were two proteins with documented kinase function (Tlk1 and Tlk2). Immunoprecipitation experiments demonstrated, as shown in Fig. 7, that an antibody against Tlk1 precipitates a renaturable protein kinase, the activity of which is down-regulated after irradiation. These results verify that Tlk1 is the protein kinase responsible for the activity we named pK90.

Discussion

Using the mentioned in-gel renaturation assay we were able to identify a band at about 90 kDa in the autoradiogram, the activity of which was down-regulated within minutes after irradiation of HL-60 cells with doses as low as 1 Gy. It is one of the shortcomings of this assay that a phosphorylation of a target protein by a protein kinase cannot be unequivocally distinguished from the adsorption of [γ^{32}P]ATP by a renaturable nucleotide binding protein. Therefore we performed a phosphoamino acid analysis to demonstrate that the observed activity results from a serine/threonine kinase.

In contrast to the molecular mass of about 90 kDa in SDS–PAGE we found that the native protein elutes from a calibrated gel-filtration column in fractions, where calibration proteins with a molecular mass of about 450 kDa were found. This indicates that pK90 exists in the nucleus in the form of a multi-protein complex. As we did not find any activity in the gel-filtration fractions around 90 kDa one can conclude that all of the pK90 activity is present in this 450 kDa complex. We have shown that pK90 activity is lost after treatment with alkaline phosphatase, indicating that the activity of the kinase is controlled by phosphorylation. It is interesting to note that the dephosphorylation reaction with alkaline phosphatase was only possible in the presence of 0.1% SDS, indicating that a dissociation of the 450 kDa complex by the detergent was necessary to allow access of the alkaline phosphatase to the kinase molecule. Experiments with other detergents (Triton X-100, NP40, and Na-deoxycholate) showed that only SDS was efficient in breaking up the complex. The very limited accessibility of the kinase in its native form was further verified by the observation that we were unable to immunoprecipitate the enzyme with the anti-Tlk1 antibody in the absence of SDS.

As shown in Fig. 4C pre-treatment of HL-60 cell with the protein synthesis inhibitor cycloheximide inhibited the radiation induced down-regulation of the pK90 activity. This cannot be explained by an inhibition of pK90 synthesis as one would expect lower level of

Table 2
Results from the MS/MS analysis of pK90

Accession No.	Name	kDa	*p*	Sequence
gi—4504167	G1 to S transition 1	56.4	10^{-52}	KVGFNPK DFPQMGR AYFETEK SVVAPPGAPK HLIVLINK KGEFETGFEK TFDAQIVIIEHK MDDPTVNWSNER
gi—6063017	Tousled-like kinase 1	82.4	10^{-10}	IDDLLR DHPTLNER AFDLYEQR
gi—11140819	Tousled-like kinase 2	86.1	10^{-6}	IDDLLR DHPTLNDR

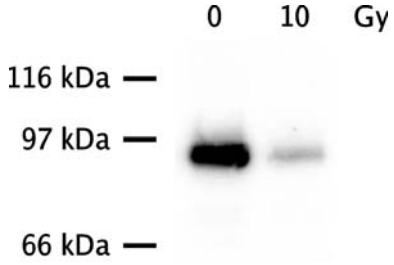

Fig. 7. In situ kinase assay of proteins immunoprecipitated with a Tlk1 antibody. The down-regulation of the renaturable kinase activity 15 min after a dose of 10 Gy verifies the mass spectroscopic finding that the renaturable kinase pK90 is identical to Tlk1.

enzyme activity if the intracellular concentration of the enzyme drops. One explanation might be the reduced synthesis and therefore the reduced intracellular activity of a protein phosphatase which is responsible for the dephosphorylation on pK90. Although this seems to be the most likely explanation, it is also possible that cycloheximide induced cellular stress leads to the activation of signal transduction pathways like activation of protein kinases like ERKs or p38 [16], which consecutively lead to a pK90 activation.

The initial design of the purification procedure proved to be difficult as ion exchange chromatography failed to give any useful separation of pK90. An explanation for this problem can be found if the titration curve of Tlk1 is considered. The curve shows that the protein has only a minimal charge at pH values ranging from about 6 up to the isoelectric point of 8.8, making a separation on an ion exchange column difficult. The shape of the titration curve of pK90 is also the reason why attempts were unsuccessful to purify the kinase by isoelectric focusing. Because of these difficulties a purification procedure consisting only of affinity chromatography steps was developed. It is obvious from Table 1 that the overall purification from this procedure is rather low. One reason for this low overall purification can be found in the in-gel renaturation assay used for the determination of the enzymatic activity. An analysis of the band, which is visible in the autoradiogram as a result of pK90 activity, revealed that it is not only caused by pK90 auto-phosphorylation but to a larger extent by the phosphorylation of other proteins present in the gel at 90 kDa. As the amount of these contaminating proteins decreases during purification, the amount of possible substrates decreases, leading to less signal than expected if the activity of the enzyme alone is taken into account.

As shown in Table 2 mass spectrometry yielded, apart from the fragments of TlK1, which was shown to be identical to pK90 by immunoprecipitation, 8 peptides belonging to a protein named G_1 to S phase transition protein 1 homolog (GST1-Hs). The identification of this GTP binding protein, which is homologous to elongation factor 1 alpha [12] together with the partially purified Tlk1, is unexpected, as GST1-Hs is considered to be a cytoplasmic protein with a molecular mass around 55 kDa. An explanation for this may be, apart from a contamination and an anomalous separation on SDS–PAGE, the existence of an hitherto un-described nuclear isoform of this protein.

The three peptides, found by MS/MS analysis, which are part of Tlk1 also fit to two other proteins. One is KIAA0137 (gi—6633952) and the other is the SNARE protein kinase SNAK (gi—7960243). An inspection of the cDNA sequence of all three proteins showed that the cDNAs are almost identical and differ only in the length of the 3′ sequence and hence in the position of the start codon. The lack of a start codon, to initiate the open reading frame in KIAA0137 and the position of the start codon in the SNARE protein kinase, which is downstream of the first ATG in the more complete Tlk1 cDNA sequence makes it, together with the migration of the immunoprecipitated Tlk1, very likely that Tlk1 is the full length protein identical to pK90.

The tousled-like kinase 1 was initially identified [24] and later on cloned [26] as the human homolog to the product of the plant gene *Tousled* (*TSL*). The product of the *TSL* gene in *Arabidopsis thaliana* was found to be a

serine/threonine kinase, which seems to be involved in plant development [21,20]. In human cells two homologs of the *TSL* gene product were identified and named Tlk1 and Tlk2 [26]. In the same paper [26] it was demonstrated that the activity Tlk1 as well as of Tlk2 is up-regulated during the S phase of the cell cycle and that the induction of DNA double-strand-breaks with agents like etoposide leads to a down-regulation of the activity of Tlk1. Very recently [9] it has been demonstrated that the activity of Tlk1 is temporarily down-regulated after exposure to ionizing radiation. In this paper [9] it was also demonstrated that Chk1, a down-stream effector kinase of ATM, phosphorylates Tlk1 on serine 695 resulting in a decrease of Tlk1 activity. Although this signal transduction pathway fits very well to our experimental results, other mechanisms, like the activation of a protein phosphatase, could be considered, if the observed inactivation of pK90/Tlk1 by alkaline phosphatase treatment is taken into account.

Acknowledgments

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