Apoptotic signalling cascade in photosensitized human epidermal carcinoma A431 cells: involvement of singlet oxygen, c-Jun N-terminal kinase, caspase-3 and p21-activated kinase 2

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Photodynamic treatment (PDT) elicits diverse cellular responses and can also cause apoptosis. In the present study the cascade of signalling events involved in PDT-induced apoptosis was investigated using Rose Bengal (RB) as the photosensitizer, and human epidermal carcinoma A431 cells as the cell model. We show that a 36-kDa kinase detected by an in-gel kinase assay is markedly activated during PDT-triggered apoptosis. Immunoblot analysis revealed that this 36-kDa kinase represents the C-terminal catalytic fragment of p21-activated kinase (PAK)2. Generation of this active fragment of PAK2 is mediated by the caspase family of proteases, which are activated by PDT. The specific caspase inhibitors (acetyl-Asp-Glu-Val-Asp-aldehyde and acetyl-Tyr-Val-Ala-Asp-chloromethylketone) block the PDT-induced caspase-3 activation and subsequent PAK2 cleavage/activation, indicating a major role for the caspase family proteases in PDT-induced apoptosis. Both PDT-induced caspase-3 activation and PAK2 cleavage/activation can be inhibited by the singlet oxygen scavengers, L-histidine and α -tocopherol, but not the hydroxyl

radical scavenger, mannitol, demonstrating that singlet oxygen is an immediate early-apoptotic signal generated by PDT. In addition, PDT can induce a two-stage activation of the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) in A431 cells; the early-stage JNK activation is singlet oxygen-dependent, whereas the late-stage JNK activation is mediated by the singlet oxygen-triggered caspase activation. Experiments using anti-sense oligonucleotides against JNK1 and PAK2 further show that during PDT-induced apoptosis the early-stage JNK activation is required for caspase activation, and that the late-stage JNK activation is regulated by the caspase-mediated cleavage/activation of PAK2. Collectively, a model for the PDT-triggered apoptotic signalling cascade with RB is proposed, which involves singlet oxygen, JNK, caspase-3 and PAK2, sequentially.

Key words: photodynamic treatment, apoptosis, JNK, PAK2.

INTRODUCTION

Photodynamic treatment (PDT) is a process in which a photosensitive dye is selectively delivered into target cells, followed by irradiation of the cells with visible light [1,2]. Interaction of the excited photosensitizer with molecular oxygen results in the formation of reactive oxygen species (ROS), such as singlet oxygen and hydroxyl radicals, which can cause severe damage to the cellular constituents and are believed to be responsible for cell destruction [3,4]. Photosensitizers include dyes, such as Rose Bengal (RB) and Methylene Blue (MB), drugs, such as tetracyclines, chlorpromazine and Photofrin, and endogenous porphyrins [5,6].

PDT can induce diverse cellular responses, including apoptosis [7]. Apoptosis (programmed cell death) plays an important role in the embryogenesis and homeostasis of multicellular organisms. Impairment of the process may cause several human diseases, including neurodegenerative disorders and cancer [8]. Apoptotic cell death is morphologically characterized by chromatin condensation, membrane blebbing and cell fragmentation [9]. These morphological changes are also accompanied by

biochemical changes, including DNA fragmentation [10]. A growing body of evidence has shown that caspases, a family of cysteine proteases identified in recent years, play a crucial role in apoptotic execution (for review see [11,12]). Caspases exist as proenzymes and can be activated during apoptosis via proteolytic processing to two smaller subunits, which then form functionally active proteases that can act on many substrates. Among the caspases, caspase-3 (also known as CPP32/Yama/apopain) has been implicated as a downstream effector protease that can be activated by other upstream caspases [13,14].

Recently, protein phosphorylation has been shown to be involved in regulating the apoptotic process. Alteration in the activities of several protein kinases can be observed during apoptosis of a variety of cell types (reviewed in [15]). Among these kinases, recent evidence has indicated that the c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK) is a key component in regulating entry into apoptosis in several cell types, under certain circumstances [16–18]. JNK, an emerging member of the mitogen-activated protein kinase (MAPK) family, can be rapidly activated by environmental stress and inflammatory cytokines [19,20]. The

Abbreviations used: Ac-DEVD-cho, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; BCA, bi-cinchoninic acid; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GSK, germinal centre kinase; GST, glutathione S-transferase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MB, Methylene Blue; MBP, myelin basic protein; MEKK1, MAPK kinase kinase 1; PAK, p21-activated kinase; PDT, photodynamic treatment; RB, Rose Bengal; ROS, reactive oxygen species; SEK1, SAPK/extracellular-signal-regulated kinase kinase 1; Z-DEVD-AFC, benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin.

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regulatory components in the JNK pathway are MAPK kinase kinase 1 (MEKK1), SAPK/extracellular-signal-regulated kinase kinase 1 (SEK1), JNK and c-Jun [21–24]. By using dominant-negative mutants of c-Jun or SEK-1 as tools, activation of the JNK pathway has been demonstrated to be required for stress-induced apoptosis in U937 leukaemia cells and for growth factor withdrawal-triggered apoptosis in PC-12 pheochromocytoma cells [16,17].

In addition to JNK, another kinase named p21-activated kinase (PAK) has been shown to be activated during apoptosis and may also be involved in the apoptotic signalling events [25–29]. PAKs are a family of 62–68 kDa serine/threonine kinases that can be activated by small (21 kDa) guanine triphosphatases, Cdc42 and Rac, which participate in regulating actin polymerization [30–32]. Three isoforms of PAK, termed α -, β - and γ -PAK (or PAK1, 3 and 2 respectively), have been identified in mammalian tissues and all have similar sequences containing an N-terminal regulatory region with a p21 binding site and a C-terminal kinase domain (for review see [33]). PAK can be activated *in vitro* by Cdc42/Rac-mediated autophosphorylation or by proteolytic removal of its N-terminal regulatory region [30,34,35]. The free C-terminal fragment of PAK can undergo autophosphorylation/activation, and the regulatory autophosphorylation site has been identified [34,36]. Although the direct downstream substrates of PAKs are largely unknown, recent studies have indicated that PAKs may act as upstream regulators of the JNK and p38 MAPK pathways. For example, recombinant JNK can be activated in *Xenopus* oocyte lysates by addition of constitutively active PAK1 [37], and overexpression of PAK1 or 2 was reported to cause activation of JNK and p38 MAPK [38,39].

Although PDT can induce apoptosis, little is known about the signalling cascades involved in this process. In the present study we characterize the PDT-induced apoptotic signalling events, using RB as the photosensitizer and human epidermal carcinoma A431 cells as the cell model. We show that activation of a 36-kDa kinase can be detected during PDT-triggered apoptosis. Activation of this kinase is found to be closely associated with caspase-3 activation in PDT-treated cells. We demonstrate that this 36-kDa kinase is in fact the C-terminal catalytic fragment of PAK2, and that the PDT-induced cleavage/activation of PAK2 is mediated by a caspase-dependent mechanism. Moreover, a two-stage JNK activation is observed in PDT-treated cells. By using singlet oxygen scavengers, caspase inhibitors and the antisense oligonucleotides against JNK1 and PAK2 as tools, the connection between signalling events involving singlet oxygen, JNK, caspase-3 and PAK2 is dissected, and a model for the PDT-triggered apoptotic signalling cascade with RB is proposed.

EXPERIMENTAL

Materials

[γ - 32 P]ATP was purchased from Amersham (Little Chalfont, Bucks., U.K.). RB, MB, Dulbecco's modified Eagle's medium (DMEM), Tween 20, L-histidine, α -tocopherol, mannitol, goat anti-rabbit and anti-mouse IgG antibodies conjugated with alkaline phosphatase were obtained from Sigma (St. Louis, MO, U.S.A.). Acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-cho), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk) and benzoyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD-AFC) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Anti-JNK1 (C17) antibody, anti- α PAK (C19) antibody and the 19-amino-acid control peptide (C19 peptide) for this antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal

Figure 1 Spectral output of the light source and the fluence rate at the surface of the cell cultures

The light source was a 120-W tungsten-halogen lamp, 30 cm above the surface of cell cultures, and the fluence rate of light, with wavelengths in the visible light region, is shown.

anti-(CPP32/caspase-3) antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). CDP-Star[®] (a chemiluminescent substrate for alkaline phosphatase) was purchased from Boehringer Mannheim (Mannheim, Germany) and bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL, U.S.A.). Protein A-Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden).

Substrates for the kinase assay

Myelin basic protein (MBP) was purified from porcine brain following a procedure described previously [40]. Glutathione S-transferase (GST)-c-Jun (residues 1–79) was expressed in *Escherichia coli* and then purified using a GSH-affinity column for the assay of JNK activity.

Antibody production

The anti-PAK2 (N17) antibody was produced in rabbits by using the peptide, MSDNGELEDKPPAPPVR, which corresponds to the N-terminal region (amino acids 1–17) of human and rabbit PAK2 [31,35], as the antigen. Peptides were coupled to keyhole-limpet haemocyanin using glutaraldehyde. The production and affinity-purification of the anti-peptide antibody were performed as described previously [27,28].

Cell culture and PDT

The epidermal carcinoma A431 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. One day prior to experiments, cells were plated on 60- or 100-mm culture dishes. PDT was performed by incubating the cells in medium, containing various concentrations of RB or MB, in the dark at 37 °C for 30 min. After incubation, cells were irradiated with a commercially available 120 W tungsten-halogen lamp from a fixed distance of 30 cm for 30 min, and the dishes were then incubated at 37 °C in a CO₂ incubator for the indicated time periods. The spectral output of the light source and the fluence rate at the surface of the cell cultures, in the visible light region, are shown in Figure 1. The cells were washed twice with ice-cold PBS and lysed in 400 μ l of solution A [20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM sodium fluoride, 20 mM sodium pyrophosphate and 1 mM sodium orthovanadate] on ice for 10 min. The cell lysates were collected and

then sonicated on ice for 3×10 s periods, followed by centrifugation at 15000 *g* for 20 min at 4 °C. The resulting supernatants were used as the cell extracts.

Immunoblots

Immunoblotting was carried out essentially as described previously [41,42]. Anti- α PAK (C19) antibody (0.2 μ g/ml), anti-PAK2 (N17) antibody (1 μ g/ml), anti-JNK1 (C17) antibody (0.2 μ g/ml) or anti-(CPP32/caspase-3) monoclonal antibody (0.25 μ g/ml) was used to immunoblot proteins transferred from SDS/polyacrylamide gels on to PVDF membranes. The proteins of interest were detected by using goat anti-rabbit or anti-mouse IgG antibodies conjugated with alkaline phosphatase and CDP-Star[®] according to the procedure provided by the manufacturer. For re-probing the same membrane with another kind of antibody, the blotted membranes were first incubated with 200 ml of stripping buffer [62.5 mM Tris/HCl (pH 6.7), 100 mM 2-mercaptoethanol and 2% (w/v) SDS] at 50 °C for 30 min with occasional agitation to strip off the bound antibodies. After washing three times in TTBS buffer [20 mM Tris/HCl (pH 7.4), 0.5 M NaCl and 0.05% Tween 20], the stripped membranes were immunoblotted with a different antibody.

In-gel kinase assay

The in-gel kinase assay was performed as previously described [27]. Briefly, 0.5 mg/ml of MBP was co-polymerized in SDS/10% polyacrylamide gels, and cell extracts (40 μ g) were resolved in these MBP-containing gels. After removing SDS from the gels, proteins were renatured, and an in-gel kinase assay was carried out by incubating the gel in phosphorylation buffer containing [γ -³²P]ATP. The gels were stained, destained and dried, and ³²P-incorporation into MBP was detected by autoradiography.

Caspase-3 activity assay

Caspase-3 activity was measured by using Z-DEVD-AFC as the fluorogenic substrate. Cell lysate (100 μ g) and 0.1 mM Z-DEVD-AFC were incubated in 250 μ l of caspase assay buffer [25 mM Hepes (pH 7.5), 0.1% CHAPS, 10 mM dithiothreitol (DTT) and 100 units/ml aprotinin] for 3 h at 37 °C. Ice-cold caspase assay buffer (1.25 ml) was then added to the mixture and the relative caspase-3 activity was determined by fluorescence spectrophotometry (Hitachi, F-2000; excitation 400 nm, emission 505 nm).

Immunoprecipitation and JNK activity assay

Before immunoprecipitation the cell extracts were first diluted to equal protein concentrations with solution A. For immunoprecipitation, 0.5 ml of cell extracts (1.0 mg/ml) was incubated with 10 μ l of anti-JNK1 (C17) antibody (200 μ g/ml) at 4 °C for 1.5 h and then with 40 μ l of Protein A-Sepharose CL-4B (30%, v/v) for another 1.5 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 1 ml of solution B [20 mM Tris/HCl (pH 7.0) and 0.5 mM DTT] containing 0.5 M NaCl, and resuspended in 40 μ l of solution B. For measuring JNK activity in the immunoprecipitates, the immunoprecipitates were incubated in a 50 μ l mixture containing 20 mM Tris/HCl (pH 7.0), 0.5 mM DTT, 0.2 mM [γ -³²P]ATP, 20 mM MgCl₂ and 0.1 mg/ml GST-cJun (1–79) at room temperature for 10 min with shaking. The reaction was stopped by the addition of 2 $\times$ Laemmli sample buffer and the samples were analysed by SDS/PAGE (12.5% polyacrylamide).

The phosphorylated GST-cJun (1–79) (approx. 37 kDa) was identified by autoradiography.

Inhibition of JNK1 and PAK2 by anti-sense oligonucleotides

JNK1 sense (5'-ATC ATG AGC AGA AGC AAG CGT GAC-3') and anti-sense (5'-GTC ACG CTT GCT TCT GCT CAT GAT-3'), and PAK2 sense (5'-ATC ATG TCT GAT AAC GGA GAA) and anti-sense (5'-TTC TCC GTT ATC AGA CAT GAT) oligonucleotides were obtained from Life Technologies (Grand Island, NY, U.S.A.). These oligonucleotides were synthesized in phosphorothioate-modified conditions and purified by HPLC. These sequences represent the amino acid codons –1 to +7 of human JNK1 and PAK2. The oligonucleotides were dissolved in 30 mM Hepes buffer (pH 7.0) and transfected into cells using LIPOFECTAMINE[™] (Life Technologies). A431 cells grown in 60-mm culture dishes were incubated at 37 °C in 1 ml of Opti-MEM I medium (Life Technologies; modified Eagle's minimum essential medium buffered with Hepes and sodium bicarbonate), containing LIPOFECTAMINE[™] (12 μ g) and oligonucleotides (35–70 μ M) for 72 h. Cells were then subjected to PDT and the cell extracts were analysed.

Analytical methods

Protein concentrations were determined using the BCA protein assay reagent from Pierce. Quantification of the relative intensity of signals detected from immunoblots and autoradiograms was performed by densitometric scanning using a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

PDT induces activation of a 36-kDa kinase in A431 cells

To investigate the kinase-mediated signalling events involved in the PDT-induced cellular responses, A431 cells pre-loaded with RB or MB were exposed to visible light, and the cell extracts were subjected to in-gel kinase assays using MBP as the substrate. RB, MB or visible light alone had no effect on the renaturable kinase activities of the cells (Figures 2A and 2B). However, combined treatment with RB (or MB) and visible light caused a dramatic activation of an approx. 36-kDa kinase in A431 cells (Figures 2A and 2B, arrow). Figure 2(C) shows the time-course of activation of the 36-kDa kinase in A431 cells by PDT with RB. It was noticed that PDT did not induce activation of this 36-kDa kinase immediately in cells, as the 36-kDa kinase activity could only be initially detected after approx. 1.5 h. The result suggests that activation of this 36-kDa kinase may participate in the signalling events of the late-stage cell responses to PDT.

Activation of the 36-kDa kinase is closely associated with PDT-induced apoptotic biochemical changes in A431 cells

In agreement with previous reports [7,43–49] that PDT with various photosensitizers can lead to death of several cell types, PDT with RB caused a significant loss in the viability of A431 cells as assessed by a Trypan Blue exclusion assay (Figure 3A). Approximately 35% and 70% of the cells died after 4 and 12 h respectively following PDT with RB. It was observed that the morphology of the A431 cells changed drastically after prolonged treatment with PDT. Changes included cell shrinkage, membrane blebbing and detachment from the culture dish, all of which are typical characteristics of apoptosis (results not shown). Since activation of the 36-kDa kinase occurred at approx. 1.5 h after PDT, it would be interesting to know the relation between this kinase activation and apoptosis induced by PDT. Therefore one

Figure 2 Activation of a 36-kDa kinase in A431 cells by PDT

(A and B) A431 cells were incubated with or without various concentrations of RB (A) or MB (B), as indicated, in the dark at 37 °C for 30 min. Cells were then left untreated or irradiated with visible light for 30 min and incubated at 37 °C for a further 2 h. Cell extracts (40 μ g) were resolved in SDS/10% polyacrylamide gels containing MBP, and the in-gel kinase activities were assayed as described in the Experimental section. (C) A431 cells were incubated with 5 μ M RB in the dark at 37 °C for 30 min. Cells were then irradiated with visible light for 30 min and incubated at 37 °C for various time periods as indicated. Cell extracts (40 μ g) were resolved in SDS/10% polyacrylamide gels containing MBP, and the in-gel kinase activities were assayed. The position of the PDT-activated 36-kDa kinase is shown by an arrow. The experiments were repeated three times and the results were reproducible.

of the prominent biochemical changes widely used to detect apoptosis, namely cleavage/activation of caspase-3, was monitored in A431 cells at various time intervals after PDT. As can be seen in Figure 3(B), cleavage of the 32-kDa caspase-3 proenzyme into its active 17-kDa subunit was clearly and initially detected at approx. 1.5 h after PDT. Caspase-3 activity assays using the fluorogenic substrate Z-DEVD-AFC further revealed a good correlation between cleavage and activation of caspase-3 following PDT (Figures 3B and 3C). The results taken together show a close association of the 36-kDa kinase activation with the PDT-induced apoptotic biochemical changes and also suggest

Figure 3 PDT induces cell death and caspase-3 activation in A431 cells

(A) A431 cells preincubated with or without 5 μ M RB in the dark at 37 °C for 30 min were irradiated with or without visible light for 30 min and then incubated at 37 °C for various time periods as indicated. At each time point, cells were detached by trypsin digestion and the viable cells were counted under microscopy after staining with Trypan Blue. The number of viable cells before PDT was taken to be 100%. The result shown here is the average of three independent experiments, and the standard deviations are all < 4%. (B and C) A431 cells subjected to PDT for various time periods, as indicated, were harvested for preparation of cell extracts. Cell extracts (60 μ g) were resolved by SDS/PAGE (15% polyacrylamide) and electroblotted on to a PVDF membrane. (B) The membrane was then probed with anti-caspase-3 antibody as described in the Experimental section. The arrow indicates the position of the active p17 subunit of caspase-3. Lane C represents control cells without PDT. (C) The same cell extracts (100 μ g) were also analysed for caspase-3 activity using Z-DEVD-AFC as the substrate. The experiments were repeated three times and the results were reproducible.

that this kinase may be involved in the apoptotic signalling events elicited by PDT in A431 cells.

Identification of the PDT-activated 36-kDa kinase as the C-terminal catalytic fragment of PAK2

It has been reported that the MAPK family members, including JNK and p38 MAPK, can be activated by PDT in murine keratinocytes [50] and human skin fibroblasts [51,52]. Because the apparent molecular mass of the PDT-activated 36-kDa kinase described in the present study is distinctly different from those of JNK (approx. 46–54 kDa) and p38 MAPK (approx.

Figure 4 Identification of the PDT-activated 36-kDa kinase as the C-terminal fragment of PAK2

(A) Recognition of the PDT-activated 36-kDa kinase by the PAK antibody. The bands containing the 36-kDa kinase activated by 1 μ M RB (or MB) plus light as described in Figures 1(A) and 1(B) were excised from the dried gels and were rehydrated in buffer [125 mM Tris/HCl (pH 6.8) and 1 mM EDTA] containing 0.1% SDS for 1 h. As negative controls, the bands corresponding to the position of 36-kDa kinase in A431 cells treated with 1 μ M RB (or MB) alone were also excised and rehydrated as described above. The rehydrated gel slices were inserted into the wells of the stacking gel of another SDS/10% polyacrylamide gel and subjected to SDS/PAGE. After electrophoresis, proteins were electroblotted on to PVDF membranes, and the blotted membranes were probed with anti- α PAK (C19) antibody (lanes 1–4). The bound antibodies were stripped from the blotted membranes, and the stripped membranes were then re-probed with anti- α PAK (C19) antibody in the presence of 1 μ g/ml of the C-terminal 19-amino-acid peptide of α PAK (lanes 5–8). Lanes 1, 2, 5 and 6 represent samples from cells treated with RB alone (lanes 1 and 5) or RB plus light (lanes 2 and 6). Lanes 3, 4, 7 and 8 represent samples from cells treated with MB alone (lanes 3 and 7) or MB plus light (lanes 4 and 8). (B and C) PDT induces cleavage of PAK2 in A431 cells. A431 cells were subjected to PDT with 5 μ M RB and then incubated at 37 °C for various time intervals as indicated. Cell extracts (60 μ g) were resolved in 10% (B) or in 12.5% (C) polyacrylamide gels and were electroblotted on to PVDF membrane. The membrane was then probed with anti- α PAK (C19) antibody (B) or anti-PAK2 (N17) antibody (C) as described in the Experimental section. Lane C represents control cells without PDT. The positions of intact PAK2 and its C- or N-terminal cleavage product are indicated by the arrowheads and arrows respectively.

42 kDa), it seems likely that this 36-kDa kinase may represent an unidentified target for PDT. Recently, we identified that a 36-kDa kinase termed autophosphorylation-dependent protein kinase (auto-kinase) is in fact a C-terminal catalytic fragment of PAK2 [36]. We also found that cleavage of PAK2, to generate a 36-kDa active catalytic fragment, can be induced in cells undergoing apoptosis triggered by environmental stresses, such as UV irradiation, heat shock and osmotic shock [27–29]. Thus it is possible that the PDT-activated 36-kDa kinase described in the present study may also represent the C-terminal catalytic fragment of PAK2. To test this possibility, the bands corresponding

Figure 5 Effects of caspase inhibitors on the PDT-induced cleavage and activation of PAK2 in A431 cells

A431 cells were preincubated with or without 300 μ M Ac-DEVD-cho (D) and/or Ac-YVAD-cmk (Y) in culture medium at 37 °C for 1 h. The cells were then subjected to PDT with 5 μ M RB and incubated at 37 °C for 2 h. Cell extracts were prepared and analysed as below. (A) Cell extracts (60 μ g) were resolved by SDS/PAGE (15% polyacrylamide) and electroblotted on to a PVDF membrane. The membrane was then probed with anti- α PAK (C19) antibody. The arrow indicates the position of the active p17 subunit of caspase-3. (B) Cell extracts (60 μ g) were resolved by SDS/PAGE (10% polyacrylamide) and electroblotted on to a PVDF membrane. The membrane was then probed with anti- α PAK (C19) antibody. The position of the 36-kDa C-terminal fragment of PAK2 is denoted by an arrow. (C) Cell extracts (40 μ g) were resolved in a MBP-containing SDS/10% polyacrylamide gel followed by the in-gel kinase assay described in the Experimental section. The position of the PDT-activated 36-kDa kinase is denoted by an arrow. The experiments were repeated three times and the results were reproducible.

to the 36-kDa kinase detected in the extracts of cells exposed to 1 μ M RB or MB plus visible light as described in Figures 2(A) and 2(B) were excised from the dried gels, re-hydrated, and subjected to SDS/PAGE followed by immunoblotting with a commercial anti- α PAK (C19) antibody, which is raised against the C-terminal peptide region of α PAK (PAK1), but can recognize all three PAK isoforms on the immunoblot. The result showed that this PDT-activated 36-kDa kinase can indeed be recognized by the anti- α PAK (C19) antibody, and inclusion of the C-terminal 19-amino-acid peptide of α PAK can prevent this recognition (Figure 4A). This finding indicated that the PDT-activated 36-kDa kinase was a member of the PAK family and may have been a catalytic fragment of PAK2. To explore further, we examined whether PDT could induce cleavage of PAK2 in cells. This was performed by immunoblotting extracts of the PDT-treated cells with the anti- α PAK (C19) and anti-PAK2 (N17) antibodies. As shown in Figure 4(B), both α PAK (PAK1; approx. 68 kDa) and PAK2 (approx. 62 kDa) were detected by

the anti- α PAK (C19) antibody in A431 cells. Besides the two bands, however, another blotted band with a molecular mass of approx. 36 kDa appeared clearly at 1.5 h after PDT (Figure 4B, arrow). When blotting with the anti-PAK2 (N17) antibody, it was found that the intensity of the 62-kDa PAK2 band decreased significantly 1.5 h after PDT (Figure 4C, arrow head), with a concomitant appearance of an approx. 30 kDa blotted band at this time point (Figure 4C, arrow). Because the anti-PAK2 (N17) antibody is raised against the N-terminal peptide of PAK2, this 30-kDa blotted band could represent the N-terminal fragment of PAK2, generated during apoptosis of A431 cells triggered by PDT. The results demonstrate that PAK2 is cleaved near the central region of the molecule to generate a pair of N- and C-terminal fragments with molecular masses of approx. 30 and 36 kDa respectively. Furthermore, the 36-kDa C-terminal fragment of PAK2 appeared to possess active kinase activity during PDT-induced apoptosis as supported by the in-gel kinase assay described in Figure 2.

Caspase inhibitors block PDT-induced cleavage/activation of PAK2 in A431 cells

It is well known that numerous proteins and enzymes are targeted for proteolysis by the caspase family of proteases during apoptosis in many cell types triggered by a variety of extrinsic and intrinsic apoptotic stimuli [11,12]. Since caspase-3 can be activated by PDT in A431 cells (Figures 3B and 3C), we investigated its possible role(s) in the induction of the biochemical changes observed during PDT-triggered apoptosis, with the help of specific tetrapeptidic caspase inhibitors, Ac-DEVD-cho and Ac-YVAD-cmk. Immunoblotting analysis showed that the PDT-induced cleavage of the 32-kDa caspase-3 proenzyme into its active 17-kDa subunit could be markedly inhibited by both inhibitors (Figure 5A), demonstrating the efficacy of the two inhibitors in blocking caspase-3 activation in A431 cells. When the same cell extracts were further subjected to immunoblotting with the anti- α PAK (C19) antibody and to the in-gel kinase assay, respectively, it was found that both inhibitors prevented PDT-induced cleavage (Figure 5B) and the subsequent activation (Figure 5C) of PAK2. It is noted that both caspase inhibitors did not interfere with the activity determination by the in-gel kinase assay since we found that direct exposure of extracts of PDT-treated cells to high dose (150 μ M) Ac-DEVD-cho and/or Ac-YVAD-cmk at 30 °C for 30 min did not change its in-gel kinase activity pattern at all (results not shown). The result indicates that the caspase family of proteases are key upstream regulators, responsible for the PDT-induced cleavage and activation of PAK2. Furthermore, this result together with the finding that cleavage/activation of PAK2 is closely associated with the activation of caspase-3 (Figures 2–4) suggest that among the caspases, caspase-3 may be an important one involved in this process, although the possible participation of other effector caspases, such as caspase-7, cannot be excluded at present.

Singlet oxygen scavengers prevent PDT-induced caspase-3 activation and the subsequent cleavage/activation of PAK2 in A431 cells

Singlet oxygen has been reported to be an important mediator in PDT-induced responses [50,51,53]. To further elucidate the mechanisms linking PDT to caspase activation, and the subsequent apoptotic biochemical changes in cells, we tested whether singlet oxygen generation during PDT was involved in this process. It has been shown that some scavengers for singlet oxygen, such as L-histidine and α -tocopherol, can inhibit the PDT-induced gene induction response and the photosensitized

Figure 6 Effects of scavengers for singlet oxygen and hydroxyl radicals on the PDT-induced caspase-3 activation and cleavage/activation of PAK2

A431 cells were incubated with L-histidine (1 mM; H), α -tocopherol (300 μ M; T) or mannitol (5 mM; M) at 37 °C for 1 h and then subjected to PDT with 5 μ M RB, followed by incubation at 37 °C for a further 2 h. Cell extracts were prepared and analysed for caspase-3 activation (A), and cleavage (B) and activation (C) of PAK2, as described in the legend to Figure 5. The experiments were repeated three times and the results were reproducible.

oxidation-mediated lipid peroxidation [53–55]. Therefore A431 cells were treated with and without L-histidine or α -tocopherol prior to PDT, and the effects of the two compounds on the PDT-induced apoptotic biochemical changes in cells were examined. It was found that both singlet oxygen scavengers potentially inhibited the PDT-induced caspase-3 activation (Figure 6A), and cleavage and activation of PAK2 (Figures 6B and 6C respectively) in A431 cells. Since PDT can generate hydroxyl radicals in cells, in addition to singlet oxygen, and most singlet oxygen scavengers are good quenchers for hydroxyl radicals [53,54], it is possible that quenching of hydroxyl radicals can also participate in the blocking effects of L-histidine and α -tocopherol described above. However, the observation that mannitol, a known hydroxyl radical scavenger, which quenches the hydroxyl radical far more specifically due to its low rate constant for reaction with singlet oxygen [54,56], had no effect on the PDT-induced caspase-3 activation (Figure 6A) and PAK2 cleavage/activation (Figures 6B and 6C), suggesting that hydroxyl radicals may not play an important role in death signalling elicited by PDT with RB.

These results taken together indicate that singlet oxygen mediates the PDT-induced activation of caspase-3 and the subsequent cleavage/activation of PAK2 in A431 cells.

Rapid and persistent activation of JNK during PDT-induced apoptosis of A431 cells

Generation of singlet oxygen is known to occur rapidly upon PDT. From the kinetic viewpoint, it is apparent that a gap of time (approx. 1.5 h) exists between rapid generation of singlet oxygen and the delayed activation of caspase-3 following PDT. Thus signalling event(s) must exist that are used to link singlet oxygen to caspase-3 activation. As mentioned above, PDT can induce JNK activation in cells [50–52]. However, the connection between JNK activation and the PDT-induced apoptotic biochemical changes remains obscure. In agreement with the previous report [50], we also observed a rapid and persistent JNK activation in PDT-treated A431 cells (Figure 7A). Visible

light or RB alone had little effect on JNK activity in A431 cells (results not shown). It is interesting to note that a fluctuation of the JNK activation level occurred during the later stages of PDT; the level of JNK activation dropped significantly at 1 h but returned to the same high level at 1.5 h (Figure 7A). To elucidate the relationship between singlet oxygen generation, JNK activation and caspase-3 activation during PDT-induced apoptosis, the time-course of JNK activation was assayed in extracts of cells pre-incubated with L-histidine or Ac-DEVD-cho before PDT. The results are summarized in Figures 7(B) and 7(C). It was found that blockage of singlet oxygen generation by L-histidine inhibited the PDT-induced early (5–30 min), as well as the late (120 min), stages of activation of JNK. However, prevention of caspase-3 activation by Ac-DEVD-cho had no effect on the PDT-induced early-stage JNK activation, but did abolish the late-stage (Figures 7B and 7C). The results provide initial evidence that a two-stage JNK activation mechanism exists in the PDT-induced apoptotic signalling events in A431 cells. The early-stage JNK activation is singlet oxygen-dependent, whereas the late-stage JNK activation is mediated by the singlet oxygen-triggered caspase activation.

Figure 7 PDT-induced JNK activation in A431 cells is regulated by singlet oxygen and caspase activation

(A) Time-course activation of JNK in A431 cells by PDT. A431 cells were subjected to PDT with 5 μ M RB and incubated at 37 °C for various time intervals, as indicated. Cell extracts were prepared and JNK1 was immunoprecipitated from the cell extracts followed by a kinase activity assay using GST-cJun (1–79) as the substrate, as described in the Experimental section. The reaction products were analysed by electrophoresis on an SDS/12.5% polyacrylamide gel and the phosphorylated GST-cJun (1–79) was identified by autoradiography. The amount of JNK1 protein from 60 μ g of cell extracts at each time point was analysed by immunoblotting, and the result is shown below the autoradiograph of the phosphorylated GST-cJun (1–79). The positions of the phosphorylated GST-cJun (1–79) and JNK1 are indicated, respectively, to the left of the panel. Lane C indicates the activity and protein level of JNK1 from control cells without PDT. (B) Differential effects of L-histidine and Ac-DEVD-cho on the PDT-induced activation of JNK in A431 cells. A431 cells were incubated with L-histidine (1 mM; H) or Ac-DEVD-cho (300 μ M; D) at 37 °C for 1 h and then subjected to PDT with 5 μ M RB, followed by incubation at 37 °C for various time intervals, as indicated. The activity assay of immunoprecipitated JNK1 and immunoblot analysis of JNK1 from cell extracts were performed, and are shown, as described above. (C) Densitometric analysis of the JNK1 activity from (B). The experiments were repeated three times and the results were reproducible.

Attenuation of the PDT-induced cleavage/activation of caspase-3 and PAK2 by the JNK anti-sense oligonucleotide in A431 cells

Because activation of the JNK pathway has been reported to be essential for apoptotic induction in some cell types by certain extracellular stimuli [16–18], it would be interesting to know whether activation of the JNK pathway is required for PDT-induced apoptosis in A431 cells. This was evaluated by incubating the cells with the JNK anti-sense oligonucleotide to inhibit its *de novo* synthesis and subsequently measuring the effect of PDT on these cells. After a 3-day treatment with the anti-sense oligonucleotide, the amount of JNK was reduced to approx. 40% of that in the control cells (Figure 8A). This effect was specific, because incubating the cells with the JNK sense oligonucleotide under the same conditions had little effect on the amount of JNK in the cells (Figure 8A). Importantly, it was found that inhibition of JNK synthesis by the anti-sense oligonucleotide resulted in a significant reduction in the cleavage/activation of caspase-3 (Figure 8B) and the subsequent cleavage (Figure 8C) and activation (Figure 8D) of PAK2 in PDT-treated A431 cells. On the other hand, these effects could not be observed in those cells treated with the JNK sense oligonucleotide (Figures 8B–8D). It was noted that the JNK anti-sense oligonucleotide had little effect on the *de novo* synthesis of caspase-3 and of PAK2 in these cells (Figures 8B and 8C, respectively). Taken together the results demonstrate that JNK is an essential regulator upstream of caspase action during the PDT-induced apoptosis of A431 cells.

Cleavage/activation of PAK2 is required for the late-stage activation of JNK during PDT-induced apoptosis of A431 cells

Since both cleavage/activation of PAK2 and late-stage JNK activation occur simultaneously and correlate well with each other after PDT (see Figures 4B, 4C and 7A for comparison), and PAK may act as one of the upstream regulators of the JNK pathway [37–39], it is reasonable to speculate that the active 36-kDa catalytic fragment of PAK2, generated via caspase activity, might be involved in regulating the late-stage JNK activation during PDT-induced apoptosis. To test this possibility, the effect of PAK2 anti-sense oligonucleotide on the PDT-triggered JNK activation was investigated. Preincubating the cells with the PAK2 anti-sense, but not the sense oligonucleotide, led to a significant decrease (approx. 50%) in the level of the PDT-

Figure 8 Effects of the JNK anti-sense oligonucleotide on PDT-induced caspase-3 activation and PAK2 cleavage/activation

A431 cells were incubated with Hepes buffer alone (Bu) or with 70 μ M JNK1 sense (S) or anti-sense (AS) oligonucleotides in the presence of LIPOFECTAMINETM for 72 h and then subjected to PDT with 5 μ M RB, followed by incubation at 37 $^{\circ}$ C for a further 2 h. (**A–C**) Cell extracts (60 μ g) were resolved by SDS/PAGE (15% or 10% polyacrylamide) and electroblotted on to PVDF membranes. The membranes were probed with anti-JNK1(C17) antibody (**A**), anti-caspase-3 antibody (**B**, upper panel), or anti- α PAK(C19) antibody (**C**). In addition, the same cell extracts (100 μ g) were analysed for caspase-3 activity using Z-DEVD-AFC as the substrate (**B**, lower panel). (**D**) Cell extracts (40 μ g) were resolved in a SDS/10% polyacrylamide gel containing MBP, and the in-gel kinase activities were assayed as described in the Experimental section. Arrows indicate the positions of the p17 subunit of caspase-3 or the 36-kDa fragment of PAK2. The relative amounts of JNK1, the p17 subunit of caspase-3 and the 36-kDa fragment of PAK2 and the relative activity of the 36-kDa kinase quantified by densitometric analysis are shown below the Figures. The experiments were repeated twice and the results were reproducible.

induced cleavage (Figures 9A and 9B) and activation of PAK2 (Figure 9C). It can be seen that in the PAK2 anti-sense oligonucleotide-treated cells the total amount of PAK2 (i.e. the intact 62 kDa PAK2 plus its 36 kDa C-terminal fragment or its 30 kDa N-terminal fragment) decreased markedly as compared with that in the control cells or in the sense oligonucleotide-treated cells (Figures 9A and 9B). In contrast, the amount of 68 kDa PAK1, a PAK isoform closely related to PAK2, did not change at all in cells treated with the two oligonucleotides (Figure 9A). The result clearly shows the efficacy and specificity of this PAK2 anti-sense oligonucleotide in blocking the *de novo* synthesis of PAK2 in A431 cells. Furthermore, because the amount of caspase-3 and its cleavage/activation pattern also did not change after treatment with the PAK2 anti-sense oligonucleotide (Figure 9D), the observed decrease in the level of PDT-induced cleavage and activation of PAK2 in the anti-sense oligonucleotide-treated cells simply reflected the reduced synthesis of PAK2 in these cells. Under this circumstance, it was found the PDT-induced late-stage JNK activation was greatly hampered (Figure 9E), demonstrating a direct involvement of cleavage/activation of PAK2 in the PDT-induced late-stage JNK activation. Since the amount of JNK did not change in the PAK2 anti-sense oligonucleotide-treated cells (Figure 9E), the result further suggested that some upstream component(s) used to control the activity of JNK are subjected to regulation by the cleaved product of PAK2 during PDT-induced apoptosis of

A431 cells. On the other hand, although the late-stage JNK activation is regulated by the cleavage/activation of PAK2, it was found that the PDT-induced early-stage JNK activation was not affected at all by the PAK2 anti-sense oligonucleotide (Figure 9F). The result seems to indicate that PAK2 does not participate in the early-stage, singlet oxygen-mediated JNK activation elicited by PDT in A431 cells.

DISCUSSION

In recent years, PDT has emerged as a promising therapeutic protocol to manage solid malignancies and non-malignant disease [57]. Studies from *in vitro* culture systems and *in vivo* animal models have shown that one of the major therapeutic effects of PDT is to trigger apoptosis of the targeted cells or tissues [7,43,44]. Recently, several signalling events were found to be associated with PDT-induced apoptosis. These included events involving activity alteration of regulatory enzymes, such as phospholipases [45], JNK and p38 MAPK [50,52], cyclin-dependent kinases [46] and caspases [47,48], and events involving generation of second messengers, such as inositol-1,4,5-trisphosphate [45] and ceramide [49]. Similarly to the cell death processes induced by many other apoptotic stimuli, activation of the caspase family of proteases appears to be an essential step for apoptotic execution in PDT-treated cells (Figures 3 and 5, and

Figure 9 Effects of the PAK2 anti-sense oligonucleotide on PDT-induced activation of JNK in A431 cells

(A–E) A431 cells were incubated with Hepes buffer alone (Bu) or with 70 μ M PAK2 sense (S) or anti-sense (AS) oligonucleotides in the presence of LIPOFECTAMINETM for 72 h and then subjected to PDT with 5 μ M RB, followed by incubation at 37 °C for a further 2 h. Cell extracts were prepared and analysed as described below. (A and B) Cell extracts (60 μ g) were resolved by SDS/PAGE (10% polyacrylamide) and were electroblotted on to PVDF membranes. The membranes were probed with the anti- α PAK (C19) antibody (A) or the anti-PAK2 (N17) antibody (B). (C) Cell extracts (40 μ g) were resolved on a SDS/10% polyacrylamide gel containing MBP, and the in-gel kinase activities were assayed as described in the Experimental section. (D, upper panel) Cell extracts (60 μ g) were resolved by SDS/PAGE (15% polyacrylamide) and were electroblotted on to a PVDF membrane. The membrane was then probed with anti-(caspase-3) antibody. (D, lower panel) The same cell extracts (100 μ g) were analysed for caspase-3 activity using Z-DEVD-AFC as the substrate. (E) JNK1 was immunoprecipitated from the cell extracts followed by a kinase activity assay using GST-cJun (1–79) as the substrate, as described in the Experimental section. The lower panel represents the immunoblot of JNK1 protein from 60 μ g of cell extracts of each sample. (F) A431 cells were incubated with Hepes buffer alone (Bu) or 35 μ M PAK2 sense (S) or anti-sense (AS) oligonucleotide in the presence of LIPOFECTAMINETM for 72 h and then subjected to PDT with 5 μ M RB, followed by incubation at 37 °C for a further 30 or 120 min. JNK1 was immunoprecipitated from the cell extracts followed by kinase activity assay using GST-cJun (1–79) as the substrate. The relative amounts of PAK2, the cleavage products of PAK2 and the p17 subunit of caspase-3, and the relative activities of the 36-kDa kinase and JNK1 quantified by densitometric analysis are shown below the Figures. The experiments were repeated twice and the results were reproducible.

[47,48]). However, little is known about the connection between the previously described PET-triggered signalling events and caspase activation. Moreover, the signalling events resulting from PDT-triggered caspase activation are also unclear. In the present study, we focus on these issues and make the following observations. First, we identify cleavage and activation of PAK2, mediated by caspase action, as a new signalling event that occurs in PDT-induced apoptosis (Figures 2–5). Secondly, generation of singlet oxygen is a prerequisite for the PDT-induced activity change of JNK, caspase-3 and PAK2 in A431 cells (Figures 6 and 7). Thirdly, PDT triggers a two-stage JNK activation in A431 cells, which can be differentiated by using a singlet oxygen scavenger and a caspase inhibitor (Figure 7). Fourthly, the early-stage JNK activation mediated by singlet oxygen is essential for caspase-3 activation (Figure 8). Finally, the activated caspase-3 in turn leads to cleavage/activation of PAK2, which is required for the subsequent late-stage JNK activation (Figure 9). On the basis of these findings, a model for the PDT-triggered apoptotic signalling cascade with RB is proposed (Figure 10).

Although we have elucidated the molecular mechanism involved in the PDT-triggered apoptosis with RB in A431 cells, an important question that remains unanswered is whether different photodynamic agents and regimes will lead to the same results. We think that different photodynamic agents and regimes might have their own unique effects on target cells, in spite of triggering common events, such as singlet oxygen generation, JNK/p38 MAPK activation and caspase activation. For instance, different photodynamic agents can target to distinct cellular compartments, such as the cytoplasm or mitochondria [58], where different signalling events may arise following visible light irradiation. In addition, even if a common signalling pathway can be activated by different PDT regimes, it may cause opposite effects on apoptosis in target cells. This is supported by reports that activation of p38 MAPK promotes apoptosis of murine LY-R leukaemic lymphoblasts induced by PDT with phthalocyanine Pc4 [59], whereas the same event protects Hela cells from apoptosis following PDT with hypericin [60]. Thus although apoptosis can be elicited by different photodynamic agents and

Figure 10 Proposed model for the apoptotic signalling cascade triggered by PDT with RB in A431 cells

Interaction of the visible light-excited photosensitizer RB (RB*) with molecular oxygen results in the formation of singlet oxygen, which in turn induces a rapid activation of the JNK pathway. Triggering of the pathway leads to activation of the caspase cascade and apoptosis. Activated caspase-3 can then act on several apoptotic substrates including PAK2. Cleavage of PAK2 releases its C-terminal catalytic fragment as a 36-kDa active kinase that can further elicit a second-stage JNK activation. Each of these signalling events can be inhibited by specific treatment as indicated. See the text for details.

regimes, the detailed apoptotic mechanism might not be the same in each case.

Using scavengers for different ROS, we show that PDT-induced activation of JNK in A431 cells is mediated by singlet oxygen. A similar finding has been reported by Klotz et al. [51] that generation of singlet oxygen is required for UVA irradiation-triggered activation of JNK in human skin fibroblasts. This indicates that singlet oxygen can be a common mediator leading to JNK activation when cells are subjected to oxidative stress by certain environmental stimuli. At present, how singlet oxygen can cause JNK activation is unknown. As JNK is phosphorylated and activated by SEK1, which in turn is phosphorylated and activated by MEKK1 [21–24], it is possible that some upstream regulator(s) of MEKK1 could be activated by singlet oxygen. Further identification of these upstream regulators is needed to clarify this point.

Although activation of the JNK pathway was reported to be essential for stress- and growth factor withdrawal-triggered apoptosis, recent evidence has implied that activation of the JNK pathway may not be a common mechanism used to regulate entrance into apoptosis. For example, it was shown that activation of JNK is not essential for Fas-mediated apoptosis in Jurkat T cells and for dexamethasone-induced apoptosis in multiple myeloma cells [61,62]. It seems that whether activation of the JNK pathway is required for triggering apoptosis will be determined by the type of apoptotic stimulus. Our observation

that caspase-3 activation induced by PDT in A431 cells is markedly attenuated by the JNK anti-sense oligonucleotide (Figure 8) indicates that activation of the JNK pathway is required for the triggering of caspase activation in PDT-treated cells. This JNK-mediated caspase activation has also been observed during apoptosis of leukaemia U937 cells induced by the anti-cancer drug etoposide [18]. These observations taken together, point out that PDT, anti-cancer drugs, environmental stresses and growth factor-withdrawal can elicit a common intracellular signalling event involving JNK activation to trigger caspase activation that subsequently leads to apoptosis. The mechanism by which activated JNK can cause caspase activation during PDT-induced apoptosis is not clear at present, and obviously warrants further investigation.

Persistent activation of JNK is detected during apoptosis induced by several death signals, and the duration of JNK activation has been suggested as a determining factor of cell fates [16,50,63]. In the present study we show that PDT can induce a two-stage JNK activation in A431 cells. Besides the identification of singlet oxygen as the primary cause leading to rapid JNK activation, we also demonstrate that a caspase-dependent mechanism is employed to maintain JNK activation at a high level during the later stages of the cellular response to PDT (Figure 7). Since the early-stage JNK activation is required for subsequent caspase activation (Figure 8), this finding points out that during PDT-induced apoptosis of A431 cells, activation of JNK occurs not only upstream but also downstream of caspase activation. Interestingly, this caspase-mediated JNK activation can also be observed during apoptosis triggered by Fas, TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)/Apo2L and calphostin C (a specific inhibitor of protein kinase C) [64–66], suggesting that it might be a rather common event involved in death signalling. This raises another intriguing question that remains to be resolved. What is the functional role of the caspase-mediated JNK activation during PDT-induced apoptosis? Because caspase activation is controlled by the upstream JNK activation (Figure 8), the possibility that the caspase-mediated JNK activation is to trigger, by a feedback mechanism, sustained caspase activation during PDT-induced apoptosis, seems attractive. However, the observation that down-regulation of PAK2 by anti-sense oligonucleotide, which can prevent the caspase-mediated JNK activation, has little effect on the activation of caspase-3 (Figure 9D) seems not to favour this suggestion.

Recently, we and others have reported that PAK2 is targeted by caspases activated by various apoptotic stimuli, including anti-Fas antibodies, tumor necrosis factor- α , ceramide and environmental stresses, such as UV irradiation, heat shock and hyperosmotic shock [25–29]. The caspase-targeting site of PAK2 is located near the centre of this kinase, which connects the N-terminal p21 binding domain and the C-terminal catalytic domain and has been demonstrated to be Asp²¹² [25]. An *In vitro* study also revealed that both caspase-3 and caspase-8 could cleave PAK2 at this site [26]. Unlike cleavage of many substrate proteins by caspases, during which the functions of the substrates are destroyed, cleavage of PAK2 by caspases appears to activate its kinase activity by removal of its N-terminal regulatory domain [25–29]. However, the functional role of the C-terminal active fragment of PAK2, generated from caspase activity, remains obscure. Recent studies have suggested that it may regulate morphological changes and JNK activity during apoptosis. Transfection of this active fragment can induce shrinkage and nucleus condensation, as well as JNK activation in target cells [26]. Another experiment showed that transfection of the dominant negative PAK1 blocked the Fas-induced JNK activation in

Jurkat T cells [64]. We show in the present study that the caspase-mediated cleavage and activation of PAK2 can also occur during PDT-induced apoptosis. Furthermore, using the anti-sense technique we provide direct evidence that one of the roles that the active fragment of PAK2 plays during apoptosis is to trigger the late-stage JNK activation in PDT-treated cells (Figure 9). It appears that cleavage/activation of PAK2 is a critical step for persistent JNK activation at a high level during PDT-induced apoptosis. Thus the active fragment of PAK2 generated by caspase action may be one of the regulatory components in the JNK pathway activated during apoptosis. This finding may explain the observation that apoptosis is delayed by transfection of the dominant-negative PAK2 (either the full-length or N-terminally truncated form) in CHO cells stably expressing a CD4/Fas chimera [26], since overexpressing the dominant-negative PAK2 would interfere with the normal activation of the JNK pathway that is required for caspase activation. How the active fragment of PAK2 causes JNK activation is unknown. It seems that some upstream regulator(s) of JNK are subjected to regulation by the active PAK2 fragment, since we have observed that inactive JNK immunoprecipitated from A431 cells without any treatment cannot be directly phosphorylated and activated *in vitro* by the purified auto-kinase, an active C-terminal fragment of PAK2 [36] (results not shown). Searching for the direct *in vivo* targets of the active PAK2 fragment, which can regulate JNK activity or other apoptotic events, is now ongoing in our laboratory.

Although we have shown that the late-stage JNK activation during PDT-induced apoptosis is controlled by the active fragment of PAK2, it is worth noting that PAK2 in its intact form is not involved in the process of the PDT-induced early-stage activation of JNK (Figure 9F). This result does not exclude the possible involvement of PAK1 or PAK3 in this process. Recently, a growing member of PAK-like enzymes, including the pleckstrin-homology-domain-containing PAKs and the germinal centre kinase (GCK) subfamily have been described (see [33] for review). Similarly to PAKs, many GCK-like enzymes have been shown to activate JNK selectively [67–70], and some of them can be activated by various forms of stress [71,72]. The intriguing possibility that the GCK-like enzymes may be potential candidates for mediating the early-stage JNK activation in PDT-treated cells remains to be examined.

In conclusion, the present study uncovers the signalling events upstream and downstream of caspase activation during apoptosis of A431 cells triggered by PDT with RB. These events are integrated to form a tightly regulated signalling pathway, through which the entrance into apoptosis in photosensitized cells can be modulated.

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