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ORIGINAL ARTICLE

The p160 nuclear receptor co-activator RAC3 exerts an anti-apoptotic role through a cytoplasmatic action

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The p160 nuclear receptor co-activators represent a family of molecules, which are recruited by steroid nuclear receptors as well as other transcription factors that are overexpressed in several tumors. We investigated the role of one member of this family on the sensitivity of cells to apoptosis. We observed that overexpression of the RAC3 (receptor-associated co-activator-3) p160 co-activator inhibits hydrogen peroxide-induced cell death in human embryonic kidney 293 (HEK293) cells. The mechanism involves the activation of anti-apoptotic pathways mediated through enhanced nuclear factor kappa B (NF-κB) activity, inhibition of caspase-9 activation, diminished apoptotic-inducing factor (AIF) nuclear localization and a change in the activation pattern of several kinases, including an increase in both AKT and p38 kinase activities, and inhibition of ERK2. Moreover, RAC3 has been found associated with a protein complex containing AIF, Hsp90 and dynein, suggesting a role for the co-activator in the cytoplasmatic nuclear transport of these proteins associated with cytoskeleton. These results demonstrate that there are several molecular pathways that could be affected by their overexpression, including those not restricted to steroid regulation or the nuclear action of co-activators, which results in diminished sensitivity to apoptosis. Furthermore, this could represent one mechanism by which co-activators contribute to tumor development.
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Introduction

Nuclear receptor co-activators, recruited by sequence-specific nuclear receptors, enhance transcriptional activation of target genes via interactions with chromatin-remodeling complexes and components of basal transcription (Peterson and Logie, 2000). There are three related 160-kDa proteins, SRC-1 (steroid receptor co-activator-1), TIF-2 (transcriptional intermediary factor) and RAC3 (receptor-associated co-activator-3), encoded by separate genes, which form the SRC or p160 family of co-activators (Xu and Qingtian, 2003). The molecular mechanism by which co-activators exert the chromatin remodeling and transcriptional activation involves the histone acetyltransferase activity that SRC-1 and RAC3 have at their C-terminal domain (Spencer *et al*., 1997; Glass and Rosenfeld, 2000) such as the recruitment of other general co-activators with enzymatic activity like CBP (CREB-binding protein)/p300, p/CAF, CARM-1 and PRMT1 (Chen *et al*., 1999; Wang *et al*., 2001).

The p160 proteins are highly homologous and were initially identified as factors that interacted with nuclear receptors in the presence of ligand and were able to enhance receptor-dependent transcriptional activation (Onate *et al*., 1995; Li *et al*., 1997). However, these proteins have been reported to enhance the activity not only of nuclear receptors but also of a number of other transcription factors (Na *et al*., 1998), including nuclear factor kappa B (NF-κB), as previously described for RAC3 (Werbajh *et al*., 2000). Moreover, we have recently demonstrated that the latter co-activator could be found associated with a protein complex containing the estrogen receptor in addition to the transcription factor and could also regulate the expression of genes involved in cell proliferation (Rubio *et al*., 2006).

NF-κB consists of dimers of proteins containing the Rel dimerization domain, being the p65 (Rel-A)/p50 hetero-complex the best characterized at present. Inactive NF-κB is associated with inhibitor of NF-κB (IκB) inhibitor proteins. Phosphorylation of IκB by specific kinases activated by extracellular signals marks IκB for degradation, thereby allowing the activation of the NF-κB complex (Ghosh and Karin, 2002). In

addition to its pivotal role in immune response and inflammation, NF-κB regulates the expression of genes that control the cell cycle and cell viability (Beg and Baltimore, 1996; Guttridge *et al*., 1999; Hinz *et al*., 1999). Although several pro-apoptotic signals such as the cytokine tumor necrosis factor-α (TNF-α), osmotic and oxidative stress could activate NF-κB, most evidence supports an anti-apoptotic role for this transcription factor (Franco *et al*., 2002).

It is well established that hydroxyl radicals generated from excess H_2O_2 have a deleterious effect not only on proteins and lipid membranes but also on DNA, causing base modifications, single- or double-strand breaks, strand excision, the crosslinking of bases, induction of the tumor-suppressor protein p53 and Bax, a protein that promotes the activation of caspase-9 and apoptosis (Gross *et al*., 1999; Hengartner, 2000). There is also considerable evidence indicating the participation of mitogen-activated protein kinases (MAPKs) in the control of cell cycle and survival (Kultz and Burg, 1998; Ono and Han, 2000; Franco *et al*., 2002), and it is well known that stress and peroxides activate several of these kinases (Ono and Han, 2000). Their role in apoptosis is controversial. While there has been a tendency to link ERK1/2 activation with cell growth or survival, and JNK and p38 activation with the induction of apoptosis, both pro- and anti-apoptotic roles have been attributed to the members of the MAPK family (Ono and Han, 2000). In addition to these kinases, it has been previously reported that the phosphoinositide 3-kinase/AKT signaling pathway is critical to many aspects of cell growth, survival and apoptosis, and its constitutive activation has been implicated in both the pathogenesis and progression of a wide variety of neoplasias (Martelli *et al*., 2006). The serine/threonine protein kinase AKT has been proposed to inhibit apoptosis by inactivating several key substrates in the apoptotic cascade such as procaspase-9, BAD and the transcription factor FKHRL-1 (Zhang *et al*., 2001).

While increased apoptosis has been associated with degenerative diseases, it is well known that impairment in apoptosis is characteristic of transformed cells. There is strong evidence implicating NF-κB activation as well as overexpression of members of p160 co-activator family in tumor development (Anzick *et al*., 1997; Iwase *et al*., 2003). Therefore, inasmuch as RAC3 as well as other p160 members and CBP function as NF-κB co-activators, it seemed reasonable to hypothesize that these molecules may contribute to tumor development through mechanisms not restricted to their action as SRCs. In the present study, we have analysed the effect of co-activator overexpression on sensitivity of human embryonic kidney 293 (HEK293) cells to H_2O_2-induced apoptosis. Here, we report that the RAC3 co-activator exerts an anti-apoptotic role not only by increasing the NF-κB activity but also by modifying the activity of several kinases, inhibiting caspases and apoptotic-inducing factor (AIF) nuclear translocation.

Results

Overexpression of RAC3 co-activator inhibits H_2O_2-induced apoptosis

To determine the role of p160 co-activator overexpression on H_2O_2-induced cell death, HEK293 cells were transiently transfected with expression vectors for SRC-1, RAC3, CBP or the empty vector and then stimulated with different concentrations of H_2O_2 for 12 or 24 h. In this cell line, the efficiency of co-activator transfection was more than 90%. As shown in Figure 1, overexpression of these molecules significantly inhibited cell death induced at 12 h by a concentration of H_2O_2 of 2 mM, which potently induced apoptosis in empty-vector control cells. Figures 1a–e show images of the apoptotic (red) cells following exposure to H_2O_2 and the effects of enforced co-activator expression. Quantitative analysis of cell death by crystal violet staining of surviving cells is shown in Figure 1f and revealed a pronounced protection from H_2O_2 lethality by each of the co-activators. These observations suggest that RAC3, as well as other p160 members, may play a role in protecting cells from oxidative injury-mediated cell death.

The functional role of RAC3 in cell survival was also analysed by transfecting cells with an expression vector for RAC3 small-interfering RNA (siRNA). In these experiments, cells were transiently transfected with the expression vector for RAC3 or the empty vector, together with or without the siRNA, after which they were exposed for 24 h to 2 mM H_2O_2. At the end of this period, apoptosis was determined by scoring red-positive cells (Biocolor kit). Figures 2a–d illustrate images of the apoptotic red cells and the diagram bars (Figure 2e) correspond to the quantitative analysis of surviving cells. These results demonstrate that inhibition of RAC3 overexpression by siRNA significantly attenuates the protective effect of the co-activator. However, siRNA had little effect in cells in which RAC3 overexpression was not present. The absence of a protective effect in cells untransfected with the expression vector for the co-activator was presumably due to the essentially undetectable levels of endogenous RAC3 co-activator, at least for the protein quantity that was used in western blot assays (Figure 2f). This is in agreement with the concept that limiting quantities of p160 co-activators are present in non-tumoral cells (Sheppard *et al*., 1998; Werbajh *et al*., 2000). In addition, this figure clearly shows increased expression levels of RAC3 in cells transiently transfected with the expression vector and the resulting inhibition by siRNA compared to extracts from transfected control cells overexpressing RAC3 as well as the breast tumor T47D cell line.

The effects of constitutive RAC3 overexpression in HEK293 stably transfected cells were subsequently investigated (Supplementary Figure 10). Taken together, these findings indicate that the RAC3 co-activator plays an anti-apoptotic role in the response of cells to H_2O_2.

Overexpression of the RAC3 co-activator contributes to the inhibition of H_2O_2-induced apoptosis through an enhanced NF-κB activity

In view of evidence that both p160 as well as CBP/p300 are co-activators of NF-κB (Sheppard *et al*., 1999;

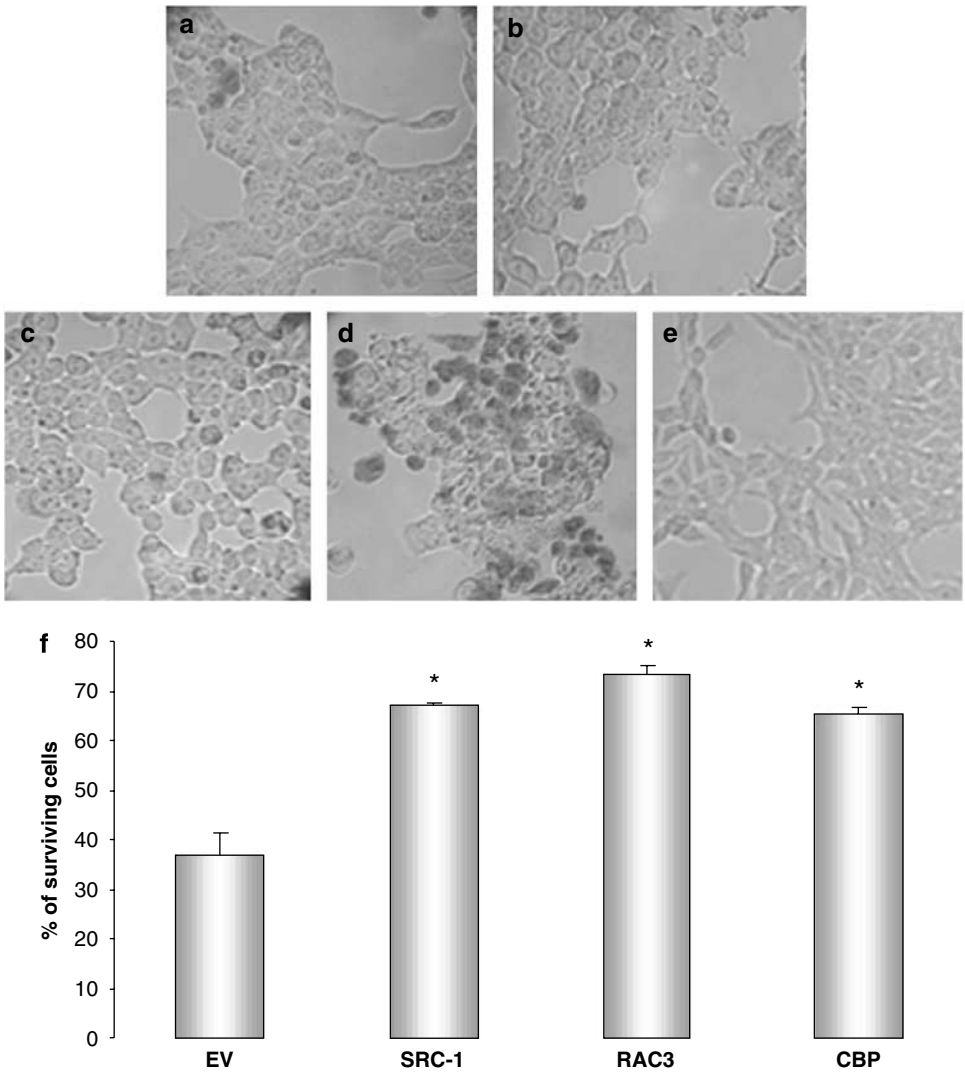

Figure 1 The overexpression of co-activators diminished sensitivity to hydrogen peroxide-induced cell death. HEK293 cells transiently overexpressing RAC3, SRC-1 or CBP were stimulated for 12 h with 2 mM of H_2O_2. Apoptosis (red cells) was determined by the apoptosis kit assay (Biocolor). (**a**) RAC3, (**b**) SRC-1, (**c**) CBP, (**d**) empty vector and (**e**) basal. (**f**) HEK293 cells overexpressing co-activators and stimulated with 2 mM of H_2O_2 for 12 h were fixed and stained with crystal violet. Absorbance of stained surviving cells was determined at 570 nm. Each value corresponds to the average of triplicate ± s.d., where the percentage of surviving cells was determined with respect to the basal absorbance. $^*P<0.001$ with respect to the control (Tukey's test). CBP, CREB-binding protein; EV, empty vector; HEK293, human embryonic kidney 293; RAC3, receptor-associated co-activator-3; SRC-1, steroid receptor co-activator-1.

Werbajh *et al*., 2000), the transcriptional activity of this transcription factor was analysed in HEK293 cells transfected with the expression vector for RAC3 and stimulated with H_2O_2. As previously described in other cell lines (Werbajh *et al*., 2000), we observed that RAC3 overexpression significantly enhanced H_2O_2-induced NF-κB transcriptional activity in HEK293 cells as compared to cells transfected with empty vector (Figure 3a). These results raise the possibility that an increase in the transcription of anti-apoptotic NF-κB target genes could represent a mechanism by which overexpression of the RAC3 co-activator protects cells from cell death. Therefore, expressions of IAP-1/2 proteins and other Bcl-2 family members were analysed in cells exposed to H_2O_2. As shown in Figure 3b, RAC3-expressing cells displayed an increase in the expression of anti-apoptotic proteins, for example, Bcl-xL, cIAP-1, cIAP-2 and Bcl-2, together with a minor regulation by H_2O_2 treatment. Because these genes could be a target of additional regulatory signals, it could explain the absence of a more significant effect via an enhanced NF-κB activity and suggests that there are other protective signals where RAC3 could be involved.

RAC3 overexpression modifies the activation pattern of several kinases implicated in cell survival regulation

There is extensive evidence showing the participation of MAPKs and AKT in the control of cell cycle progression and survival (Ono and Han, 2000). To determine if

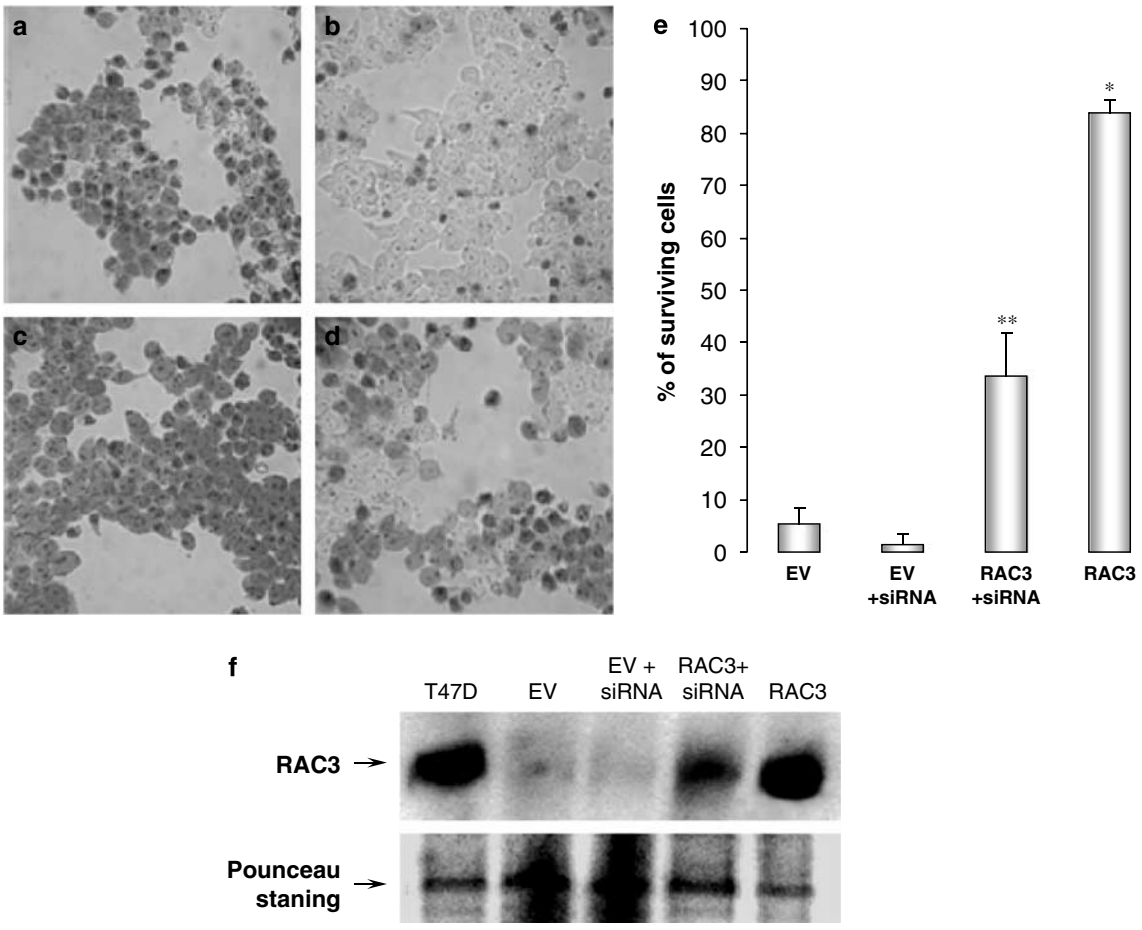

Figure 2 RAC3 anti-apoptotic effect may be specifically reverted by a RAC3 siRNA. Cells transiently transfected with pCMX-RAC3 or the empty vector, in the presence or absence of the siRNA, were stimulated with 2 mM of H_2O_2 for 24 h. Apoptosis was determined as in Figure 1. (**a**) Empty vector, (**b**) RAC3, (**c**) empty vector plus siRNA and (**d**) RAC3 plus siRNA. (**e**) A quantitative analysis was performed by counting the dead red cells at different fields, and results were expressed as percent of surviving cells. $*P<0.001$ with respect to the empty vector and $**P<0.001$ with respect to the RAC3 (Tukey's test). (**f**) Western blot of HEK293 cell extracts previously transfected as indicated. Extracts from T47D were used as positive control. HEK293, human embryonic kidney 293; EV, empty vector; RAC3, receptor-associated co-activator-3; siRNA, small-interfering RNA.

increased expression of RAC3 would induce a specific pattern of MAPK and AKT activation, expression levels of several of these proteins and their phosphorylated active forms were analysed by western blot in cells transiently transfected with the co-activator expression vector or empty-vector controls.

As shown in Figure 4, AKT activation is detected after 30 min of stimulation with H_2O_2 and remained elevated at 120 min in control cells. However, basal and stimulated levels of phospho-AKT were clearly higher in cells overexpressing RAC3. In contrast, the total amount of AKT was not affected by co-activator overexpression. Similar results were obtained when phospho-p38 expression was monitored at 60 min after H_2O_2 stimulation. In contrast, H_2O_2 exposure strongly activated ERK2 after 60–120 min but had essentially no effect on cells transfected with RAC3 expression vector. As noted in the case of AKT, total ERK1/2 protein levels remained unaffected by the enhanced RAC3 expression. Taken together, all these results indicate that overexpression of RAC3 in HEK293 cells increases the activation of NF-κB (Figure 3), AKT and p38 MAPK (Figure 4) in response to H_2O_2, and blocks ERK1/2 activation, events that may contribute to the diminished sensitivity of RAC3-expressing cells to H_2O_2-induced apoptosis.

To determine the role of these signal transduction pathways on the protective action of RAC3, we performed experiments on surviving cells by using the specific inhibitors of each kinase and NF-κB activation pathway. In these experiments, cells overexpressing or not overexpressing the co-activator were preincubated with the p38 inhibitor SB202190, the inositol-AKT inhibitors wortmannin or LY294002 and the NF-κB inhibitor sulfasalazine (Franco *et al*., 2002) before the addition of H_2O_2. As shown in Figure 5a, all the inhibitors significantly reduce the RAC3-protective effect, suggesting that all these pathways are required for an optimal anti-apoptotic action of the co-activator.

Figures 5b and c show that the concentration of inhibitors used effectively inhibit each one of the kinases and NF-κB activity.

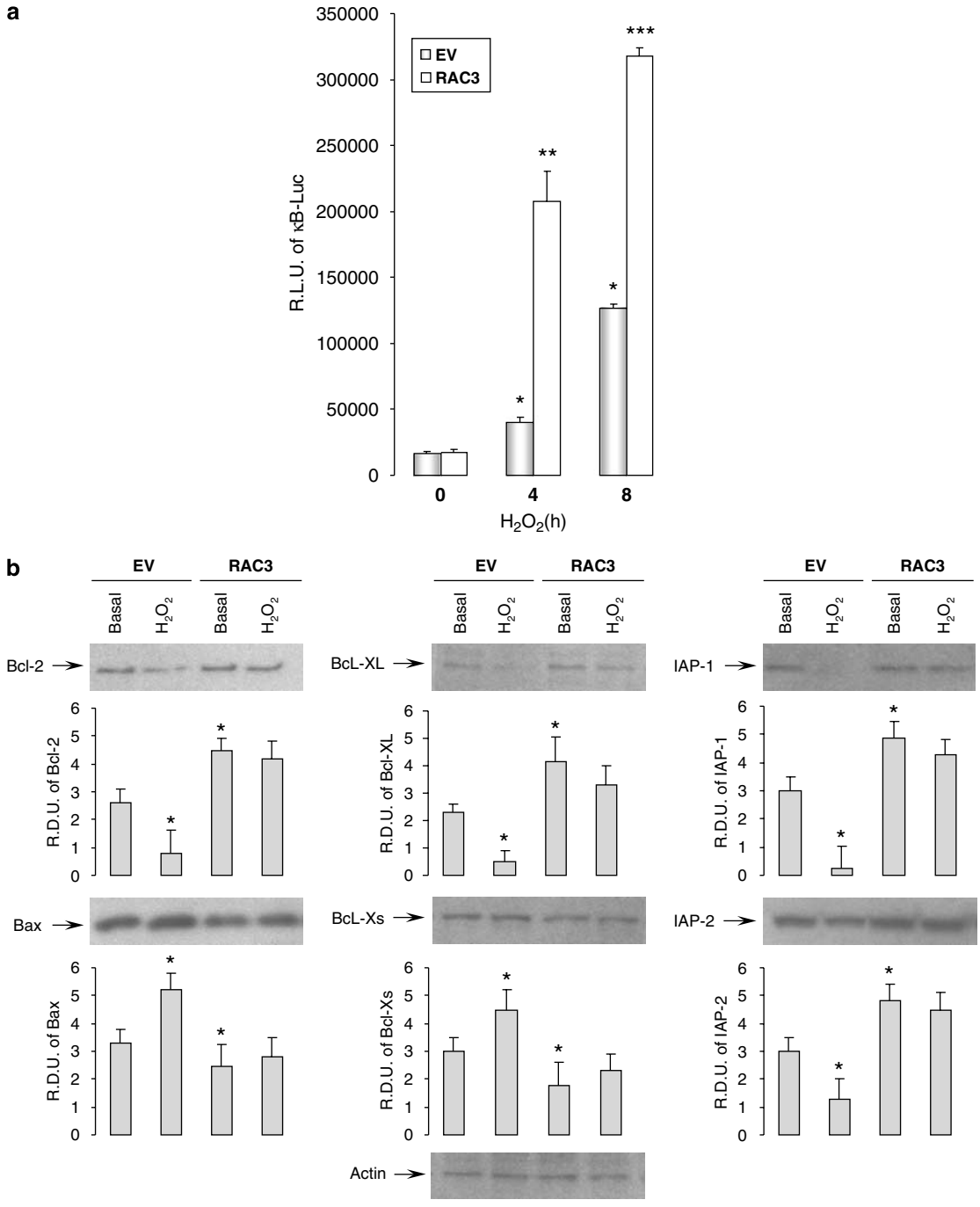

Figure 3 Role of RAC3 on NF-κB transactivation and NF-κB target gene expression. (**a**) Cells transiently transfected with a κB-Luc reporter plasmid plus RSV-βGal and pCMX-RAC3 or the empty vector were stimulated with 1 mM of H_2O_2 for 4 or 8 h. Relative luminescence units are the average of triplicate ± s.d. and were normalized with the corresponding β-galactosidase values. $*P<0.001$ with respect to EV 0 h (basal condition), $**P<0.001$ with respect to EV 4 h and $***P<0.01$ with respect to EV 8 h (Tukey's test). Similar results were obtained with different doses and hours of stimulation. (**b**) Western blot from total cell extracts was performed using the specific antibodies in HEK293 stimulated with 2 mM of H_2O_2 for 8 h, as indicated. RDU corresponds to the average ± s.d. of three independent experiments. $*P<0.01$ with respect to EV basal condition (Tukey's test). EV, empty vector; HEK293, human embryonic kidney 293; NF-κB, nuclear factor kappa B; RAC3, receptor-associated co-activator-3; RDU, relative densitometric unit.

Overexpression of RAC3 co-activator inhibits caspase-9 activation

It has been previously reported that peroxides induce cell death by activating the apoptotic mitochondrial pathway with the subsequent activation of caspase-9 (Franco *et al*., 2002; Katoh *et al*., 2004). We therefore analysed the effect of co-activator overexpression on H_2O_2-induced caspase-9 activation in HEK293 cells. As shown in Figure 6, the expression level of active caspase-9 (cleavage fragment) was increased after 24 h of H_2O_2

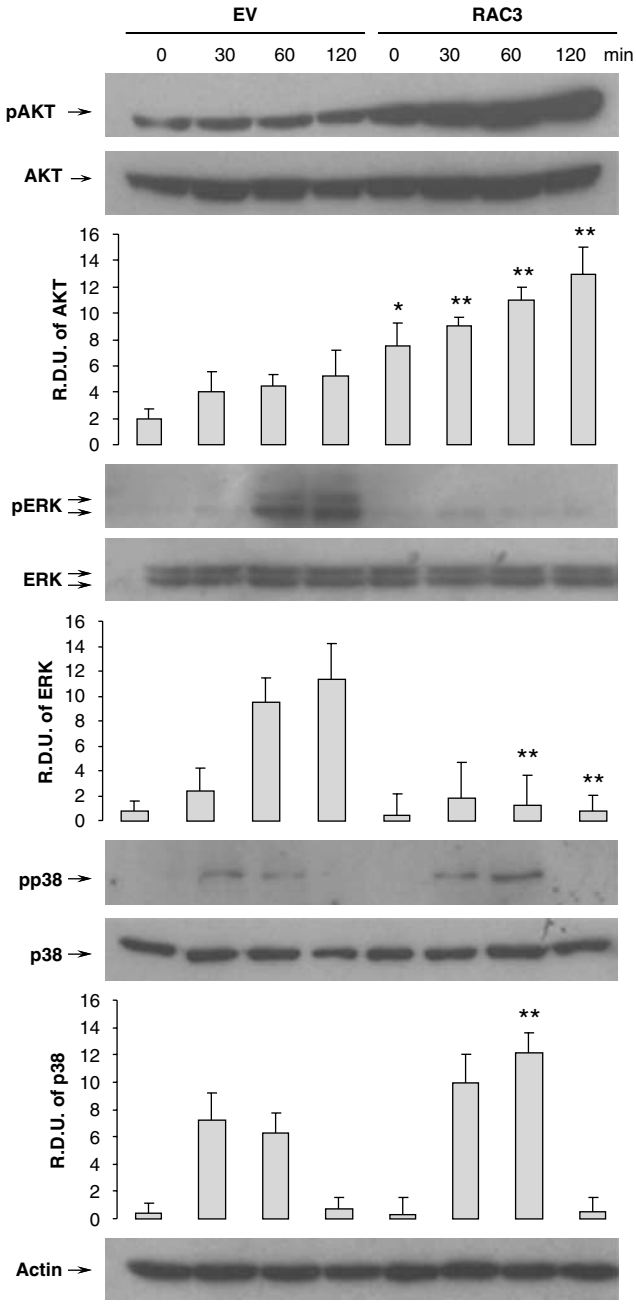

Figure 4 RAC3 overexpression regulates the activity of kinases AKT, ERK and p38. Cells transiently transfected with the pCMX-RAC3 or the empty vector were stimulated for the indicated time with 2 mM of H_2O_2. Western blot was performed using specific antibodies. RDU corresponds to the average ± s.d. of three independent experiments. $^*P<0.01$ with respect to EV at 0 h and $^{**}P<0.01$ with respect to the EV at the corresponding same time (Tukey's test). EV, empty vector; RAC3, receptor-associated co-activator-3; RDU, relative densitometric unit.

treatment in cells transfected with the empty vector, as expected. However, this activation was inhibited in cells transfected with the RAC3 expression vector. Similar results were obtained when we analysed activation of the effector caspase-3, which also correlated with diminished caspase-9 activation and reduced apoptosis with RAC3 overexpression. These observations suggest that the co-activator protects cells from peroxide-induced apoptosis by the inhibition of caspase-9 activation.

RAC3 shows a cytoplasmatic localization but translocates to the nucleus after H_2O_2 stimulation

In this study, we found some effects of RAC3 overexpression that are probably not specifically related to its well-known nuclear action as co-activator. Such is the case for the change in the pattern of kinase activation. Therefore, we analysed the subcellular localization of this co-activator in basal conditions and after peroxide treatment.

As shown in Figure 7, RAC3 is mainly localized at cytoplasm in basal conditions, and part of the pool of these molecules migrates to the nucleus after H_2O_2 treatment and remains nuclear for at least 8 h.

To determine if this translocation could be associated with microtubule machinery, we also studied the effect of microtubule polymerization inhibitor colchicine. We found that under colchicine treatment, the RAC3 translocation is completely inhibited, indicating the requirement of a functional microtubule-associated transport (Figure 7a). Similar results were obtained when we analysed the AIF subcellular localization (Figure 7b).

RAC3 is physically associated with AIF in a protein complex containing the Hsp90, the immunophilin (FKBP52) and the microtubule-associated motor protein dynein

It has been previously reported that H_2O_2-induced cell death involves the release of AIF from mitochondria and its import to the nucleus (Susin *et al*., 1999; Arnoult *et al*., 2002) and that Hsp70 specifically interacts with AIF and antagonizes apoptosis (Ravagnan *et al*., 2001; Gurbuxani *et al*., 2003). In addition, it is well known that Hsp70 is usually associated with Hsp90, which is involved in the regulation of signaling protein function and trafficking together with immunophilins and the microtubule-associated motor protein dynein (Pratt and Toft, 2003; Galigniana *et al*., 2004).

In view of these reports and our current findings concerning the RAC3 effects, the subcellular localization and the microtubule-associated machinery requirement, we decided to investigate the possible interaction of RAC3 with AIF and the Hsp90-immunophilin-based molecular machinery of movement. The co-immunoprecipitation assays shown in Figure 8a demonstrate a physical interaction among RAC3, AIF and Hsp90 after H_2O_2 treatment. Moreover, all of these proteins were found to be physically associated as part of a complex containing at least the immunophilin FKBP52 and the microtubule-binding protein dynein (Figure 8a). This is in agreement with the previously described interaction among FKBP52, Hsp90 and dynein, a complex where the immunophilin acts as an anchor or adaptor that links the cargo protein complex to be transported by the microtubule machinery via dynein motor proteins (Galigniana *et al*., 2004).

To confirm the physical interaction between RAC3 and AIF, we also analysed the colocalization of these

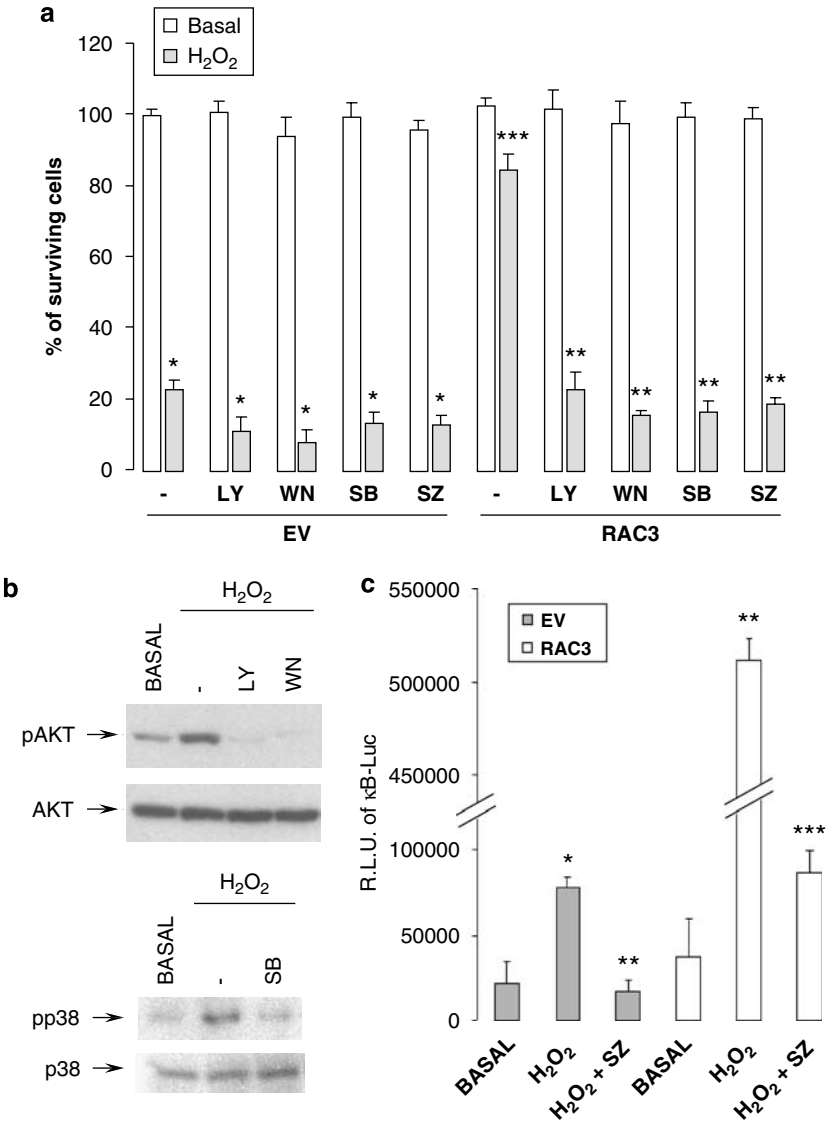

Figure 5 AKT, p38 and NF-κB activation pathways are required for an optimal RAC3 anti-apoptotic action. (**a**) Cells transiently transfected with pCMX-RAC3 or the EV were preincubated for 30 min with the inhibitors of p38, SB202190 (SB; 20 μM); AKT, wortmannin (WN; 100 nM) or LY294002 (LY; 50 μM) and the NF-κB inhibitor sulfasalazine (SZ; 400 μM) and then stimulated with 2 mM of H_2O_2 for 24 h. Surviving cells were stained with crystal violet, and absorbance of stained surviving cells was determined at 570 nm. Each value corresponds to the average of triplicate ± s.d., where the percentage of surviving cells was determined with respect to the basal absorbance. $*P<0.01$ with respect to the H_2O_2 treatment with EV, $**P<0.001$ with respect to the H_2O_2 treatment with RAC3 overexpression and $***P<0.001$ with respect to the H_2O_2 treatment with EV (Tukey's test). (**b**) Cells transiently transfected with pCMX-RAC3 or the EV were preincubated for 30 min with the inhibitors of p38, SB202190; AKT, wortmannin or LY294002 and the NF-κB inhibitor sulfasalazine and then stimulated with 2 mM of H_2O_2 for 30 min for western blot or 8 h for reporter assays. (**c**) Relative luminescence units are the average of triplicate ± s.d. and were normalized with the corresponding β-galactosidase values. $*P<0.01$ with respect to the basal condition, $**P<0.001$ with respect to the H_2O_2 treatment with EV, $***P<0.001$ with respect to the H_2O_2 with RAC3 overexpression (Tukey's test). EV, empty vector; NF-κB, nuclear factor kappa B; RAC3, receptor-associated co-activator-3.

proteins by confocal microscopy in cells stimulated with H_2O_2. Because the detection level of endogenous RAC3 was really low, in these experiments, we decided to work with cells transiently transfected with RAC3 expression vector. Figure 8b shows that both molecules are able to physically interact in the cytoplasm, once AIF was released from the mitochondria even after 8 h, when most of the cell population are not apoptotic and RAC3 could be found in the nucleus. However, in a few cells that are shown to be apoptotic (one of them is shown in the figure), a clear AIF and RAC3 nuclear localization is shown, although no colocalization of these molecules is observed. Similar results were obtained when AIF and RAC3 subcellular localization was analysed in cells that naturally overexpress the co-activator (Supplementary Figure 11).

Similar results were obtained when we studied the subcellular localization of Hsp90 and the interaction with AIF.

Interestingly, the AIF · Hsp90 complex dissociates at the peripheral nuclear space when the cells are

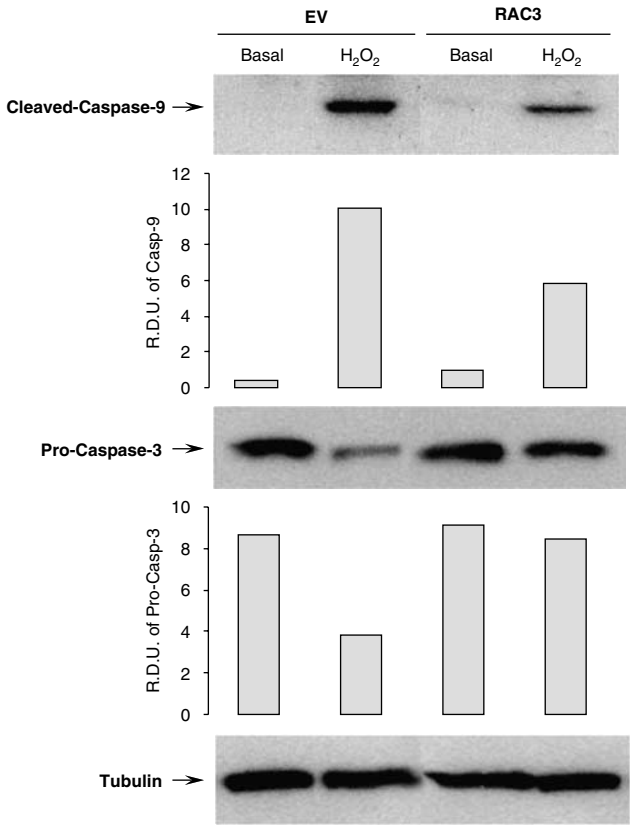

Figure 6 RAC3 overexpression inhibits caspase-9 and -3 activation. HEK293 cells transiently transfected with pCMX-RAC3 or the empty vector were stimulated for 24 h with 2 mM of H_2O_2. Western blot of total cell extracts using antibodies against the active caspase-9 and the procaspase-3, as indicated. Relative densitometric unit (RDU) is indicated. Similar results were obtained from four independent experiments. EV, empty vector; HEK293, human embryonic kidney 293; RAC3, receptor-associated co-activator-3; RDU, relative densitometric unit.

apoptotic. Thus, while AIF becomes nuclear, Hsp90 concentrates in a perinuclear area (Figure 8c). This might be due to the release of the cargo protein from the molecular machinery of movement when the nuclear pore is reached.

Overexpression of RAC3 co-activator inhibits AIF nuclear translocation

To determine whether co-activator overexpression modifies H_2O_2-induced AIF translocation, AIF localization was analysed in HEK293 cells overexpressing or not overexpressing RAC3 following stimulation with H_2O_2 for 8 h. In agreement with the results that are shown in Figure 8, confocal fluorescent microscopy images (Figure 9a) show that AIF is primarily cytoplasmic in basal conditions and translocates to the nucleus upon stimulation with H_2O_2. This translocation is impaired in cells overexpressing RAC3. This observation was confirmed by western blotting AIF in the nuclear extracts of the cells (Figure 9b). Densitometric analysis indicates that the relative densitometric unit (RDU) of AIF for H_2O_2-treated cells is almost twofold the RDU of control cells, whereas the total level of protein used in the assay was the same (see Ponceau staining under the figure).

These findings raise the possibility that attenuation of H_2O_2-mediated AIF nuclear translocation may contribute to the protective effects of RAC3.

Discussion

Two basic mechanisms by which signals may contribute to tumor growth include an increase in cell proliferation and/or a reduction in the cell death rate. In the present study, we have found that overexpression of RAC3 nuclear receptor co-activator protects cells from apoptosis induced by H_2O_2. In view of recent evidence that oxidative damage (that is ROS production) can serve a tumor-suppressor function (Takahashi *et al*., 2006), these findings raise the possibility that deregulation of the RAC3 nuclear receptor can promote tumorigenesis.

Overexpression of p160 members has been found in several tumors such as breast and ovarian cancer (Anzick *et al*., 1997; List *et al*., 2001), endometrial carcinomas (Glaeser *et al*., 2001), gastric cancers (Sakakura *et al*., 2000), prostate cancers (Gnanapragasam *et al*., 2001) and other types (Yan *et al*., 2006).

Although these molecules were originally identified as nuclear receptor co-activators, their role as co-activators of several transcription factors has been clearly demonstrated. This is the case for NF-κB (Sheppard *et al*., 1999; Werbajh *et al*., 2000), which is a key anti-apoptotic transcription factor that controls the cell cycle and cell proliferation (Beg and Baltimore, 1996; Guttridge *et al*., 1999; Hinz *et al*., 1999). Collectively, these observations suggest that p160 co-activators may play a role in oncogenesis, possibly through mechanisms unrelated to nuclear receptor activity or hormone-stimulated growth (List *et al*., 2001; Cavarretta *et al*., 2002). Thus, it is possible that p160 nuclear receptor co-activators and CBP could contribute to tumor development in the absence of steroid hormone stimulation through a mechanism that affects various survival signaling pathways that regulate cell death decisions.

RAC3 knockout mice resulted in growth retardation, small size, reduced reproductive function and mammary development (Wang *et al*., 2000; Xu *et al*., 2000). Interestingly, a recent study involving RAC3 knockout mice demonstrated that this co-activator could exert divergent effects on cell proliferation, depending on the cellular context, ranging from proliferative and tumorigenic effects in breast cells to antiproliferative effects in lymphoid cells (Coste *et al*., 2006). The latter findings are in concordance with the present results obtained with transfected HEK293 cells, in which RAC3 and other co-activators clearly displayed an anti-apoptotic role.

Previous evidence suggests a potential functional redundancy between members of the p160 family (Torchia *et al*., 1997). The present observations suggest that each of the p160 members, in addition to CBP, exert a similar role inhibiting cell death when it is

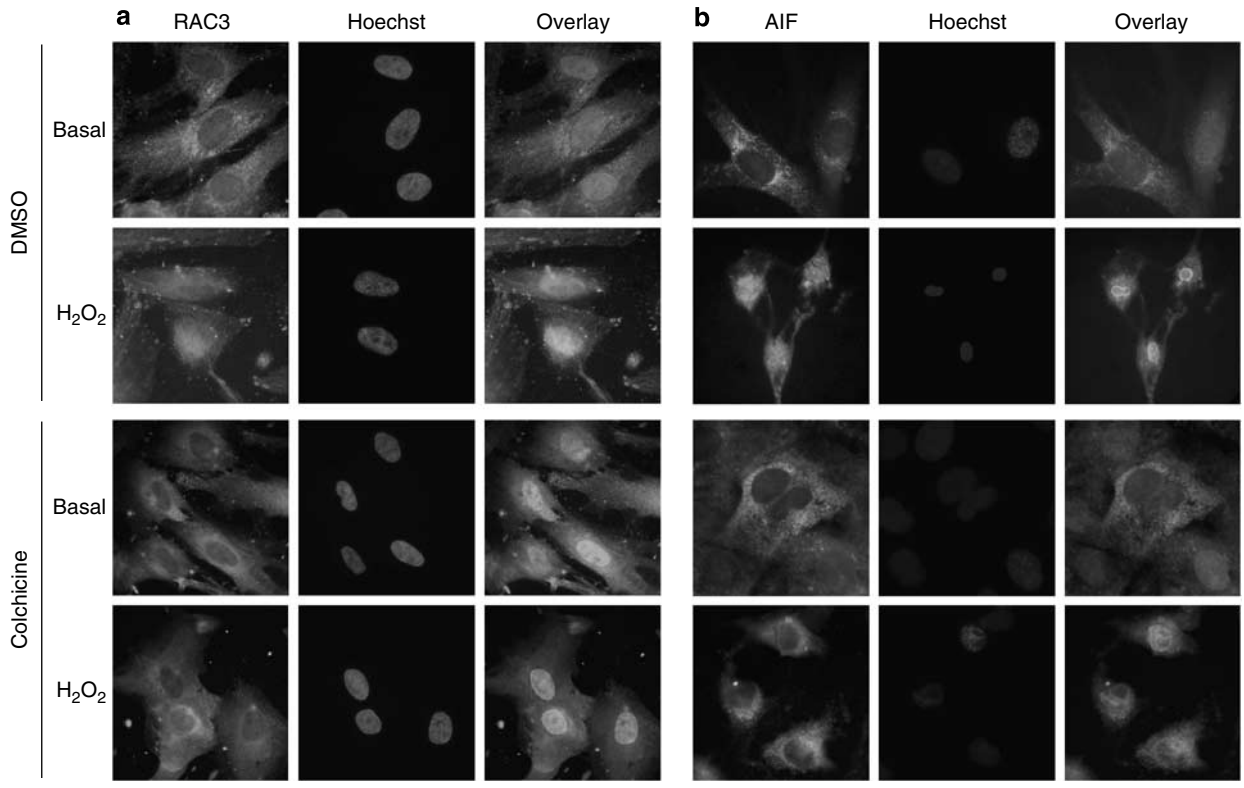

Figure 7 Inhibition of microtubule polymerization inhibits RAC3 and AIF nuclear translocation. RAC3 (**a**) and AIF (**b**) were determined by immunofluorescent staining after 8 h of H_2O_2 treatment, and colchicine 1 μM was added 1 h before peroxide stimulation. AIF, apoptotic-inducing factor; RAC3, receptor-associated co-activator-3.

overexpressed in cells displaying essentially undetectable basal levels. This is in agreement with the concept of functional redundancy. However, whether the mechanisms employed by each co-activator to exert a cytoprotective role are identical to that of RAC3 remains to be established.

It is well known that RAC3 is an NF-κB co-activator (Werbajh *et al*., 2000). Subsequently, an increase in NF-κB transactivation and expression of anti-apoptotic NF-κB target genes represent plausible mechanisms underlying attenuation of cell death. Consistent with this notion, analysis of NF-κB activity revealed enhanced transactivation following RAC3 overexpression, as anticipated. In addition, expression levels of several Bcl-2 family members, which are classical intermediates in the apoptosis control, have shown to be differentially regulated by RAC3 overexpression. While these proteins are NF-κB target genes, there are targets of additional signals, and the absence of a more pronounced effect could reflect a balance stemming from the cross-talk that exists among different signaling pathways. In addition, enhanced expression of other anti-apoptotic NF-κB target genes cannot be excluded.

In addition to the well-described role of p160 proteins in promoting transcriptional activity and gene expression, the present results suggest that RAC3 overexpression may exert additional, non-nuclear functions. These include alterations in the intracellular traffic of apoptotic regulatory proteins, such as inhibition of AIF nuclear localization, as well as caspase-9 activation. Such results suggest that RAC3 may exert certain additional non-classical co-activator activities that are involved in the control of cell death, perhaps involving the nuclear translocation control of certain proteins or, alternatively, the modulation of specific cytoplasmic actions. In this context, it has been previously reported that caspase-9 is inhibited by AKT, a kinase also involved in several additional anti-apoptotic signals (Zhang *et al*., 2001). In agreement with previous findings, we found enhanced AKT activity in cells overexpressing RAC3 and diminished sensitivity to H_2O_2-induced apoptosis. Analogously, others have reported that RAC3 overexpression may regulate cell size and growth in a steroid-independent manner, through the enhancement of AKT activity (Zhou *et al*., 2003). Such findings are in agreement with previous observations that *in vivo* RAC3 overexpression induces aberrant mammary gland development, mammary adenocarcinomas and a variety of other tumors with high frequency. Furthermore, these tumors showed increased insulin-like growth factor-I (IGF-I) levels and increased activation of the IGF-I receptor (IGF-IR)/phosphoinositide 3-kinase/AKT pathway (Torres-Arzayus *et al*., 2004). Collectively, these findings suggest that the cytoprotective effects of RAC3 overexpression may be mediated in part by the activation of the AKT pathway.

The present results also indicated that p38 kinase activation is required for the RAC3 protective effect.

These observations are in accordance with a predominance of an anti-apoptotic route through the increase in the NF-κB activity and the action of this kinase over this transcription factor (Franco *et al*., 2002). Although ERK activation is primarily associated with cell survival (Cobb *et al*., 1991; Kultz and Burg, 1998), we found reduced activity of this kinase in cells overexpressing RAC3. Such findings are in agreement with results of other studies suggesting that under some circumstances, ERK exists as a pro-apoptotic pathway (Park *et al*., 2005). These observations should be viewed in the context of the occasionally conflicting roles that MAPKs are believed to play in survival signaling (Downward, 1998; Kultz and Burg, 1998; Ono and Han, 2000; Franco *et al*., 2002; Park *et al*., 2005).

The effect of RAC3 over the caspase-9 pathway could be the consequence of both a classical co-activator action that changes the expression pattern of the NF-κB-dependent anti-apoptotic target genes as well as the non-classical effects on the activation of several kinases whose mechanism remains to be determined. However, concerning the effect over AIF translocation, our experiments suggest that there is a direct participation of RAC3 that is not related to its nuclear co-activator action.

Although there is not much evidence concerning the mechanism of AIF subcellular trafficking, and the specific mechanism by which RAC3 inhibits the AIF nuclear localization is not clear, in this study, we demonstrate that both molecules translocate to the nucleus after H_2O_2 stimulation as part of a protein complex containing Hsp90 and the immunophilin FKBP52, which are in turn associated with the microtubule-trafficking machinery (Pratt and Toft, 2003; Galigniana *et al*., 2004). In view of these observations, we may speculate that high levels of RAC3 may interfere with the structure of the protein complex, perhaps by down-titration or squelching of some components that are required for nuclear translocation, as well as with the recruitment of molecules that cooperates with a cytosolic retention. In this regard, it has been demonstrated that the interaction of Hsp70 with AIF antagonizes its apoptotic action and involves cytosolic retention (Ravagnan *et al*., 2001; Gurbuxani *et al*., 2003).

In summary, the present results demonstrate that overexpression of nuclear receptor co-activators exerts a protective role in H_2O_2-induced apoptosis. In the case of RAC3, this cytoprotective role appears to operate through multiple mechanisms, including those that are not restricted to previously well-described nuclear activities. Taken together, these findings indicate that diverse molecular pathways unrelated to steroid control can be affected by nuclear receptor co-activator deregulation and that these events are associated with

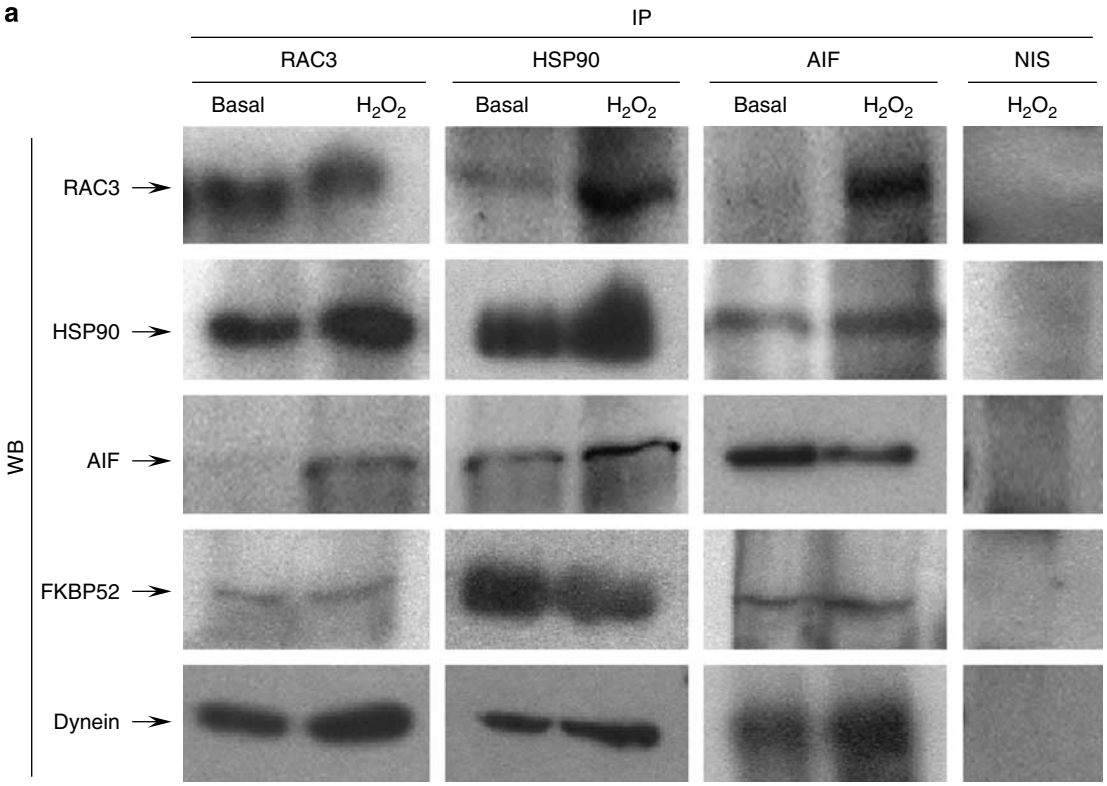

Figure 8 AIF and RAC3 are part of a protein complex associated with the microtubule-transporting machinery. (**a**) Western blot analysis of IPs from HEK293 cells stimulated with or without H_2O_2 by 8 h. NIS-IP corresponds to the non-immune serum immunoprecipitations. Confocal microscopy showing the colocalization of AIF with RAC3 (**b**) and with Hsp90 (**c**) in cells transiently transfected with pCMX-RAC3 and stimulated with H_2O_2 for 4 or 8 h. In addition to the non-apoptotic cell, one apoptotic cell where AIF is expected to be found in the nucleus is shown. AIF, apoptotic-inducing factor; HEK293, human embryonic kidney 293; IP, immunoprecipitates; RAC3, receptor-associated co-activator-3.

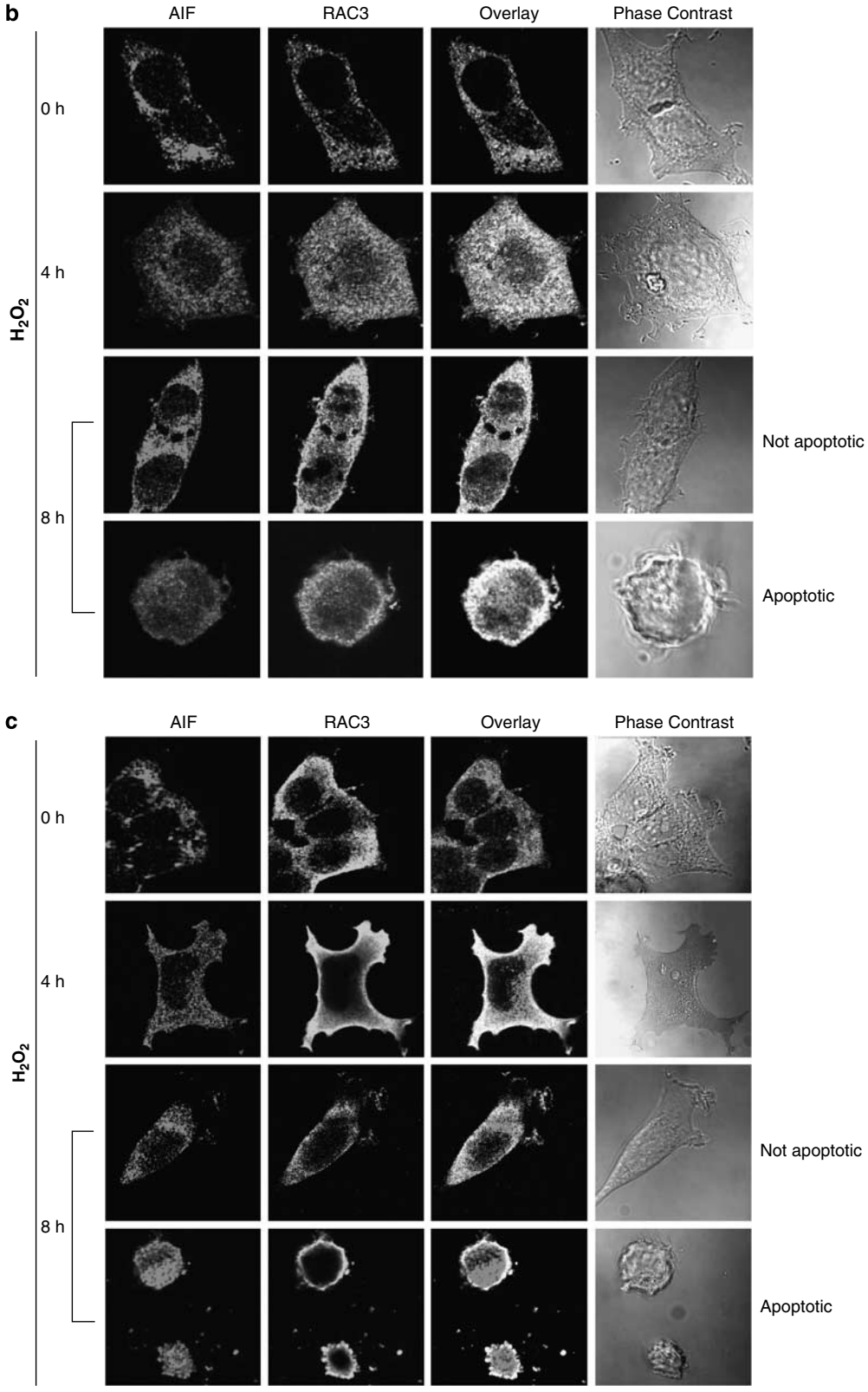

Figure 8 Continued.

diminished sensitivity to apoptosis, which could represent a mechanism by which the RAC3 co-activator contributes to tumor development. Finally, our observations suggest that in addition to steroid nuclear receptor cascades, several additional pathways might be appropriate for therapeutic intervention.

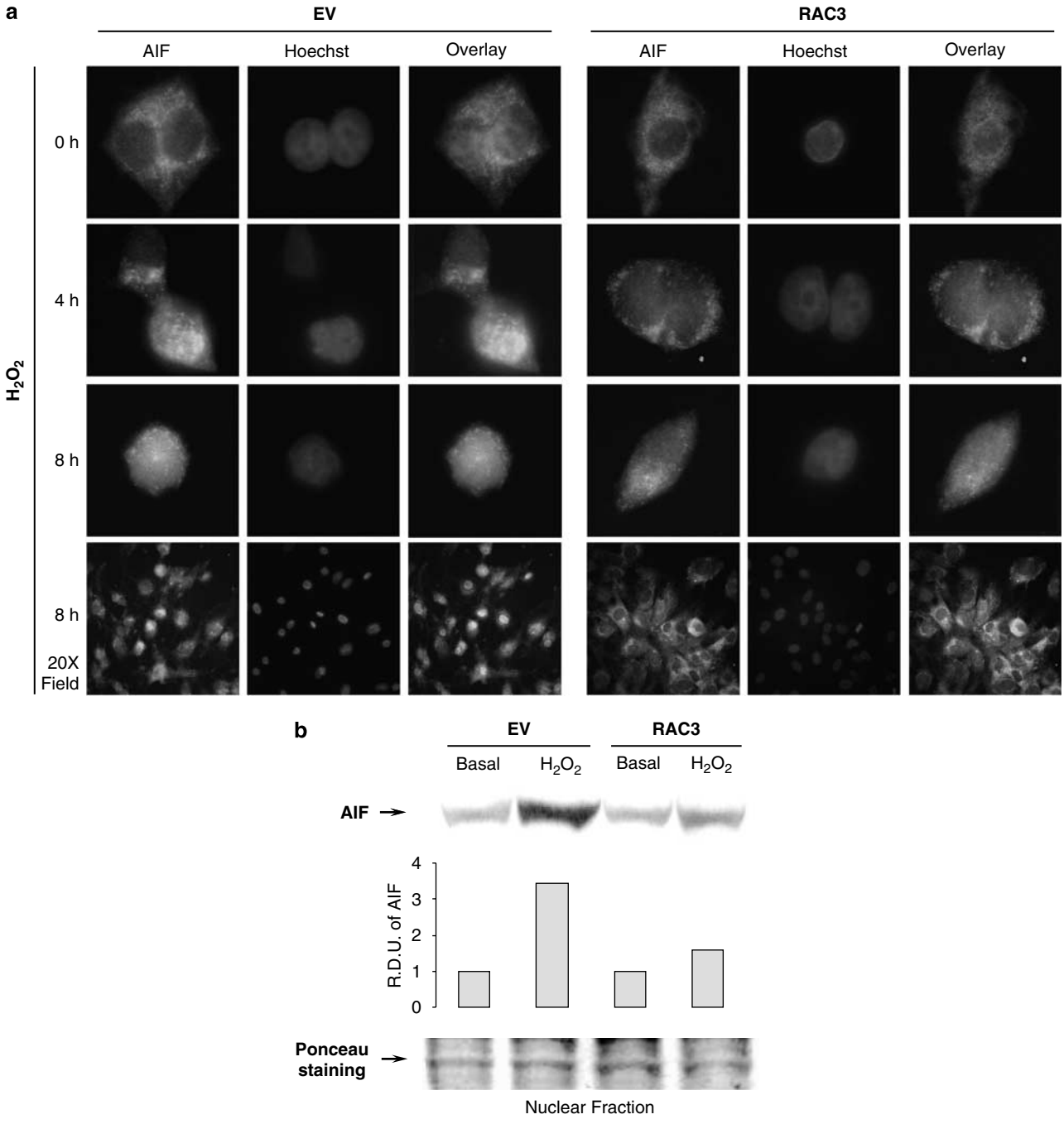

Figure 9 (**a**) AIF translocation was determined by inmunofluorescent staining after 4 or 8 h of stimulation using a fluorescein-conjugated antibody and nucleus staining with Hoechst reactive. (**b**) Western blot of nuclear extracts prepared after 24 h of stimulation with 2 mM of H_2O_2 was performed using the specific antibodies against AIF. RDU was determined with respect to the unspecific bands detected by Ponceau staining. Similar results were obtained from three independent experiments. AIF, apoptotic-inducing factor; EV, empty vector; RDU, relative densitometric unit.

Materials and methods

Cells and reagents

HEK293 cells were grown in Dulbecco's modified Eagle's medium high glucose (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), penicillin (100 U ml^{-1}) and streptomycin (100 mg ml^{-1}). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Unless stated, reagents were obtained from Sigma Chemical Co, Pharmacia or Calbiochem.

Transfection and determination of NF-κB activity

Transfection was performed as previously described (Costas *et al*., 2000; Werbajh *et al*., 2000). Briefly, HEK293 cells were cultured in 24-well plates at a density of 5×10^4 cells per well in Dulbecco's modified Eagle's medium and transiently transfected by using calcium chloride method with a total of 5.5 μg of DNA (including 1 μg of κB-Luc reporter for assays of NF-κB activity, 0.5 μg of RSV-β-Gal and pCMX (empty vector) or pCMX-RAC3) for determination of NF-κB activity. H_2O_2 was added after 24 h of transfection. The assays for

luciferase and β-galactosidase activity were performed using the appropriate substrates following the manufacturer's protocols (Promega Corp.).

For experiments on surviving cells, the cells were transfected with 5 μg of pCMX or pCMX-RAC3 or SRC-1 or CBP in the presence or absence of pSilencer-RAC3-siRNA expression vector or the scrambled control sequence, and 5 h later the medium was replaced by fresh Dulbecco's modified Eagle's medium. After 24 h, cells were re-plated to 96-well microtiter plates for cytotoxicity assays and stimulated 24 h later. The efficiency of transfection for HEK293 was determined by counting the X-gal-stained blue cells and was 97%. Some clones stably overexpressing members of p160 family were selected by subculturing the cells on a medium containing G418 antibiotic.

All the co-activator expression vectors have been previously described (Werbajh *et al*., 2000), while the pSilencer-RAC3 siRNA plasmid was prepared using the Ambion system following the manufacturer's protocol according to the previously reported sequence (Zhou *et al*., 2003).

Cytotoxicity and apoptosis assays

Cytotoxicity was determined as previously described (Costas *et al*., 2000). Briefly, cells were plated at 1.5×10^3 cells per well in 96-well microtiter plates and stimulated with different concentrations of H_2O_2 after preincubation or not for 30 min with kinases or NF-κB inhibitors, as indicated in the figure legends. After 12 or 24 h, HEK293 cells were fixed with 0.2% glutaraldehyde/2% formaldehyde and stained with 0.5% crystal violet. The absorbance corresponding to the stained surviving cells was determined at 570 nm. The percent of surviving cells was determined with respect to the basal conditions.

Apoptosis was analysed using the Apoptosis Biocolor kit following the instructions of the manufacturer.

Analysis of caspases, kinases, and pro- and anti-apoptotic proteins

The expression of active caspase-9, the levels of the pro- and anti-apoptotic proteins and kinase activity were analysed by western blot as previously described (Franco *et al*., 2002).

Briefly, for experiments involving caspase-9 and pro- and anti-apoptotic genes, cells were stimulated with or without H_2O_2 for 8 or 24 h. For experiments involving ERK2 and AKT activity, cells were stimulated for different periods of time (30, 60 and 120 min). Then, the cultures were washed with phosphate-buffered saline (PBS) and lysed at 4 °C in a lysis buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5%) containing the protease inhibitors, 10 μg ml^{-1} leupeptin, 10 mg ml^{-1} aprotinin, 1 mg ml^{-1} pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. In some experiments, we prepared subcellular fractions and lysed cells at 4 °C with 1 ml of hypotonic buffer (10 mM Tris-Cl (pH 6.7), 0.2 mM $MgCl_2$) containing a cocktail of protease inhibitors. Samples were centrifuged at 1500 r.p.m. for 10 min at 4 °C to remove the cytosolic fraction. The nuclei fraction was washed with PBS, lysed with lysis buffer and then centrifuged at 12 000 r.p.m. for 5 min at 4 °C and the supernatant was stored as the nuclear fraction.

Cell extracts were denatured in Laemmli loading buffer at 95 °C for 5 min. The protein samples were electrophoresed in a 12% SDS–polyacrylamide gel electrophoresis gel. The proteins were transferred to immunoblot polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated with the specific antibodies (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Specific proteins were detected using enhanced chemiluminescence (ECL) reagents.

In the case of kinase assays, the phosphatase inhibitors 0.5 mM of sodium fluoride and 0.5 mM of sodium vanadate were also added to the lysis buffer.

Intracellular localization of AIF and RAC3 was also observed by immunofluorescence. Briefly, after stimulation, the fixed and blocked samples were incubated with the specific AIF or RAC3 antibody overnight, and a fluorescein-conjugated goat anti-mouse secondary antibody was then added after 1 h.

Physical association of proteins

The physical association of RAC3 with AIF, Hsp90 and dynein was analysed by co-immunoprecipitation assays. Briefly, HEK293 cells were stimulated with 2 mM H_2O_2 for 24 h and then lysed in radioimmunoprecipitation assay lysis buffer containing the protease inhibitors. Supernatants of lysates were incubated overnight at 4 °C with the specific antibodies (Santa Cruz Biotechnology Inc.) and immunoprecipitated for 2 h at 4 °C with GammaBind G Sepharose (Pharmacia Biotech, USA). After six washes, Sepharose-bound immunocomplexes were separated on 6 or 12% SDS–polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene difluoride membranes. Western blot was performed as described before by using the specific antibodies (Santa Cruz Biotechnology Inc.).

These interactions were also analysed by confocal microscopy. Briefly, HEK293 cells were seeded onto six-well plates over 12-mm glass plates at a density of 2.5×10^5. After 24 h, the cells were stimulated with 2 mM H_2O_2 for 4 or 8 h. Cells were washed with PBS and fixed with 3% formaldehyde, 0.2% glutaraldehyde for 15 min. Samples were washed with PBS and then permeabilized with 0.2% Triton X-100 for 15 min. Blocking was made with 3% BSA in PBS for 1 h and washed with PBS. Primary antibody incubations were made overnight at 4 °C in PBS with 3% BSA. Samples were washed with PBS and then incubated with a fluorescein isothiocyanate (FITC) or tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated secondary antibodies for 1 h. After washing with PBS, the samples were mounted over glass plates in PBS/glycerol 1:1 solution. Samples were visualized with a confocal microscope, and digital images were taken with a digital camera coupled to the microscope. Images were analysed with Carl Zeiss LSM Image software.

Abbreviations

AIF, apoptotic-inducing factor; CBP, CREB-binding protein; IκB, inhibitor of NF-κB; p53, tumor-suppressor protein; RAC3, receptor-associated co-activator-3; SRC-1, steroid receptor co-activator-1; TIF-2, transcriptional intermediary factor-2; TNF-α, tumor necrosis factor-α.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).