Inactivation of the Inhibitory κB Protein Kinase/Nuclear Factor κB Pathway by Par-4 Expression Potentiates Tumor Necrosis Factor α-induced Apoptosis*

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Par-4 is a novel protein identified in cells undergoing apoptosis. The ability of Par-4 to promote apoptotic cell death is dependent on the binding and inactivation of the atypical protein kinases C (PKCs). This subfamily of kinases has been reported to control nuclear factor κB (NF-κB) through the regulation of the IκB kinase activity. NF-κB activation by tumor necrosis factor α (TNFα) provides a survival signal that impairs the TNFα-induced apoptotic response. We show here that expression of Par-4 inhibits the TNFα-induced nuclear translocation of p65 as well as the κB-dependent promoter activity. Interestingly, Par-4 expression blocks inhibitory κB protein (IκB) kinase activity, which leads to the inhibition of IκB phosphorylation and degradation, in a manner that is dependent on its ability to inhibit λ/PKC. Of potential functional relevance, the expression of Par-4 allows TNFα to induce apoptosis in NIH-3T3 cells. In addition, the down-regulation of Par-4 levels by oncogenic Ras sensitizes cells to TNFα-induced NF-κB activation.

The atypical protein kinase C (PKC) subfamily of isozymes (σPKCs) has recently been the focus of considerable attention. It is composed of two members, σPKC and λ/PKC (1), which appear to be involved in a number of important cellular functions including cell proliferation and survival (2-6). The mechanisms whereby the σPKCs control these functions most probably involve the ERK cascade (7-12) and NF-κB (4, 11, 13-17), since both signaling pathways are targeted by the atypical PKCs and play critical roles in cell growth and apoptosis (3, 18-25). In this regard, the σPKCs selectively bind to, and are inhibited by, Par-4, which was initially identified by differential screening in cells that were undergoing apoptosis (3, 4, 26). Consistently, the ectopic expression of Par-4 in NIH-3T3 cells induces apoptotic cell death in a manner that is dependent on its ability to block the atypical PKCs (3, 4). In addition, recent studies show that the expression of Par-4 sensitizes prostate cancer and melanoma cells to apoptotic stimuli (27), as well as that Par-4 may be a mediator of neuronal apoptosis (28). Therefore, the study of the mechanisms of action of Par-4 and the atypical PKCs may be of great importance for the understanding of the signaling events involved in programmed cell death.

Because the atypical PKCs regulate ERK (see above) and this kinase has been shown to be important in cell survival (3, 19-22), the mechanisms whereby Par-4 induces apoptosis may at least in part involve this pathway. However, this may not be the only way for Par-4 to induce apoptosis. Thus, NF-κB that is a target of the atypical PKCs (4, 11, 13-17) is a recognized anti-apoptotic molecule (18, 23-25). Therefore, it is conceivable that the impairment of the NF-κB pathway through the inhibition of the atypical PKCs may contribute to the pro-apoptotic actions of Par-4. TNFα, although it shares some components of the Fas signaling cascade, is an interesting example of the activation by a single cytokine of two different pathways with opposite effects. Thus, TNFα triggers the caspase-8 route to apoptosis and simultaneously activates NF-κB, which is an important survival signal (for a recent review, see Ref. 29, and references therein). Therefore, TNFα induces apoptosis if the NF-κB pathway is inactivated (18, 23-25). The most classical form of NF-κB is a heterodimer of p50 and p65 (Rel A) (30-32), that is sequestered in the cytosol by IκB, which prevents its nuclear translocation and activity (32, 33). Upon cell stimulation by inflammatory cytokines such as TNFα or IL-1, which are potent activators of the atypical PKCs (17, 34, 35), IκBα is phosphorylated in residues 32 and 36, which trigger the ubiquitination and subsequent degradation of IκB through the proteasome pathway (33). These events release NF-κB, which translocates to the nucleus where it activates several genes involved in cell survival and inflammation (30-33). Recently, two IκBα kinases (IκKα and IκKβ) have been identified that phosphorylate residues 32 and 36 of IκBα, and whose activity is potently stimulated by TNFα and IL-1 (36-40). Results from this laboratory demonstrated that the atypical PKCs are critical regulators of the IκK activity, which offers a mechanistic explanation to the proposed role of these PKCs on NF-κB activation (41).

The studies reported here address the possibility that Par-4...
potentiates TNF-induced apoptosis by inhibiting NF-κB through the blockade of the αPKC-IKK signaling cascade.

**MATERIALS AND METHODS**

**Plasmids, Cell Culture, and Transfections**—The HA-tagged expression plasmids for α/ PKC, Par-4, Par-4-NLS and Par-4-ΔNLSΔA, and myc-Par-4 have previously been described (3, 4). HA-tagged RasV12 was generously provided by M. White. The Flag-IKKβ and IKKα constructs were kindly provided by D. Goeddel (Tularik, Inc.). The GST-αIkBα and GST-IκBα(κBκB) plasmids (42) and the ecotropic virus packaging line Phoenix were kindly provided by D. Goeddel (Tularik, Inc.). The GST-IκBα plasmids (42) and the ecotropic virus packaging line Phoenix were kindly provided by D. Goeddel (Tularik, Inc.).

**RESULTS**

**Par-4 Inhibits the Nuclear Translocation of p65 in Response to TNFa**—Since NF-κB has been shown to have anti-apoptotic properties (see above), it was of great interest to determine the effect of Par-4 expression on the nuclear translocation of p65, as a direct measure of NF-κB activation. Par-4 was recently reported to bind the nuclear protein WT1 (44), implying that Par-4 can modulate not only the αPKCs, which are located in the cytosol (45), but also a nuclear activity. However, we have recently demonstrated that the possible nuclear function of Par-4 is not required for apoptosis (3). Thus, Par-4ΔNLS, a mutant that lacks the predicted nuclear localization signal located at amino acids 24–29 of the human Par-4 gene, was always in the cytoplasm of expressing cells and is at least as potent as the wild type protein to induce apoptosis (3). NIH-3T3 cells were then transfected with an HA-tagged version of Par-4ΔNLS, after which they were either untreated or stimulated with TNFa for 20 min. Afterward, cells were analyzed by double immunofluorescence microscopy with the monoclonal anti-HA antibody 12CA5 to detect the expressed Par-4 and a goat anti-myc antibody. The expression of Par-4 inhibits the translocation of p65 in response to TNFα. Thus, in the field shown in this figure, the cell that does not express the Par-4 construct displays a clear translocation of p65 to the nucleus, whereas the one that expresses Par-4 shows that parameter completely abrogated. When several cell fields were analyzed, it becomes clear that at least more than 70% of the TNFα-activated cells that express the ectopic Par-4 construct display an impaired nuclear translocation of p65 (Fig. 1B). Similar results were obtained when this experiment was performed with the full-length Par-4 protein (Fig. 1, C and D). It should be noted that no translocation to the nucleus of transfected full-length Par-4 was observed in response to TNFα stimulation in these experiments (Fig. 1C). This is further confirmed in the data of Fig. 1E. In that experiment, NIH-3T3 cells were either untransfected (upper panel) or transfected with the HA-tagged versions of full-length (middle panel) or ΔNLS (lower panel) Par-4 expression vectors. Afterward, cells were either untreated or stimulated with TNFα for different times, and the levels of endogenous Par-4 or of the ectopically expressed Par-4 constructs, were determined by cytosolic and nuclear fractions with the corresponding antibodies. No specific localization of Par-4 to the nucleus was detected under these conditions (Fig. 1E). Collectively, these results suggest that Par-4 is a cytosolic modulator of the NF-κB pathway. To further support this conclusion, NIH-3T3 cell lines (either control or stably overexpressing Par-4) were stimulated with TNFα for 30 min, after which cells were fractionated and the translocation...
tion of p65 to the nucleus was determined by immunoblot analysis. Results of Fig. 1 confirm that the expression of Par-4 dramatically inhibits the TNFα-induced nuclear translocation of p65.

**Par-4 Inhibits the Activation of a κB-dependent Reporter by TNFα**—To further show the implication of Par-4 in NF-κB signaling, NIH-3T3 cells were transfected with a κB-dependent luciferase reporter plasmid along with increasing concentrations of ΔNLS (Fig. 2) or full-length (data not shown) Par-4 expression vectors. Afterward, cells were either untreated or stimulated with TNFα for different times, and the levels of endogenous Par-4 or the ectopically expressed Par-4 constructs were determined in cytosol and nuclear fractions with the corresponding antibodies. Essentially identical results were obtained in another two experiments. F, NIH-3T3 cell lines either control (pBabe) or stably overexpressing Par-4 (pBabe-Par-4) were either untreated or stimulated with TNFα for 30 min, after which cells were fractionated and the translocation of p65 to the nucleus was determined by immunoblot analysis (upper panel). The immunoblot of the lower panel demonstrates the overexpression of Par-4 in the pBabe-Par-4 cell line. Essentially identical results were obtained in another two experiments.

**Fig. 1. Par-4 expression inhibits the nuclear translocation of p65.** A–D, NIH-3T3 cells were transfected with either an empty plasmid or an expression vector for HA-tagged versions of Par-4ΔNLS (A and B) or full-length Par-4 (C and D). Twenty-hours after transfection, cells were either untreated or stimulated for 20 min with TNFα (30 ng/ml), after which they were analyzed by double immunofluorescence confocal microscopy with the monoclonal anti-HA antibody 12CA5 to detect the expressed Par-4 construct (A and C, right panels) and a goat polyclonal anti-p65 antibody (A and C, left panels). A and C are representative fields of one of the experiments; B and D show the mean ± S.D. of several fields (300 cells) from three independent experiments. The arrows indicate the cells that are expressing the ectopic Par-4. E, NIH-3T3 cells were either untransfected (upper panel) or transfected with the HA-tagged versions of full-length (middle panel) or ΔNLS (lower panel) Par-4 expression vectors. Afterward, cells were either untreated or stimulated with TNFα for 30 min, after which cells were fractionated and the translocation of p65 to the nucleus was determined by immunoblot analysis (upper panel). The immunoblot of the lower panel demonstrates the overexpression of Par-4 in the pBabe-Par-4 cell line. Essentially identical results were obtained in another two experiments.
increasing concentrations of the Par-4 expression plasmid. Afterward, cells were stimulated or not with TNFα and the activity of IKKβ was determined. The expression of Par-4 (Fig. 3A) or Par-4ΔNLS (Fig. 3B) dramatically inhibits IKKβ activation by TNFα. We have previously shown that the atypical PKCs selectively target IKKβ but not IKKα (41). Of note, the expression of Par-4 did not affect TNFα-stimulated IKKα activity (Fig. 3C).

Expression of an Active λ/PKC Mutant Blocks the Inhibition by Par-4 of IκB Phosphorylation and Degradation—To demonstrate that Par-4 inhibits IKK through the atypical PKCs, an activated λ/PKC mutant (λ/PKC(C43A)) was introduced into NIH-3T3 cells either alone or together with Par-4, using recombinant replication-deficient retroviruses. Cells were either untreated or stimulated with TNFα for different times and the degradation, and Ser18 phosphorylation of IκBo was determined as a direct measure of the IKK activity in vivo. Fig. 4 shows that the expression of Par-4 impairs the phosphorylation and degradation of IκB in response to TNFα. In addition, the expression of a λ/PKC active mutant inhibits to a large extent

**Fig. 2.** Par-4 expression inhibits the κB-dependent promoter activity. A, NIH-3T3 cells were transfected with different concentrations of plasmid pCDNA3-HA-par-4ΔNLS along with 500 ng of the κB enhancer-directed luciferase reporter plasmid (κB-luc) or a constitutive CMV-directed luciferase reporter (CMV-luc). After 4 h, the DNA-containing medium was removed and cells were incubated for 16 h in serum-containing medium. Following stimulation with TNFα (30 ng/ml; 4 h), extracts were prepared and the luciferase activity was determined as a direct measure of the IKK activity in vivo. Twenty hours after transfection, cells were either untreated or stimulated with TNFα (30 ng/ml) for 7 min. Afterward, Flag-IKKβ was immunoprecipitated and its activity was determined as described under “Materials and Methods.” Essentially identical results were obtained in another two experiments. B, subconfluent cultures of NIH-3T3 cells in 100-mm plates were transfected with Flag-IKKβ (10 μg) along with 20 μg of either HA-Par-4 expression vector or control plasmid. Twenty-hours after transfection, cells were either untreated or stimulated with TNFα (30 ng/ml) for 7 min. Afterward, Flag-IKKβ was immunoprecipitated and its activity was determined as above. Essentially identical results were obtained in another two experiments. C, subconfluent cultures of NIH-3T3 cells in 100-mm plates were transfected with Flag-IKKα (10 μg) along with 20 μg of either HA-Par-4 expression vector or control plasmid. Twenty hours after transfection, cells were either untreated or stimulated with TNFα (30 ng/ml) for 7 min. Afterward, Flag-IKKα was immunoprecipitated and its activity was determined as above. Essentially identical results were obtained in another two experiments.
the blockade by Par-4 of this parameter (Fig. 4). Collectively, these findings provide independent evidence, other than the use of dominant negative mutants or antisense oligonucleotides, that the stimulation of the atypical PKCs by oncogenic Ras accounts for a substantial part of NF-κB activation.

Sensitization to the TNFα Apoptotic Actions by Expression of Par-4—The inactivation of the NF-κB pathway has been shown to be a key element in the sensitization of cells to the cell killing properties of TNFα (see above). Because Par-4 expression provokes the inhibition of NF-κB through the aPKC-IKK pathway, we determined in the following experiments if the expression of Par-4 was sufficient to allow the induction of apoptosis by TNFα. Par-4 was introduced into NIH-3T3 cells either alone or in combination with the λ/PKC active mutant using recombinant replication-deficient retroviruses as above. Afterward, cells were treated with TNFα either in the absence or in the presence of FCS, and cell viability was determined. TNFα was unable to induce cell death either in the absence or in the presence of FCS (Fig. 5). The expression of Par-4 induced apoptosis in cells that were incubated in the absence but not in the presence of FCS (Fig. 5). Interestingly, TNFα potently induced apoptosis in cells expressing Par-4 irrespective of the presence of FCS (Fig. 5). The co-expression of the λ/PKC active mutant completely abrogated cell death by TNFα in the Par-4-expressing cells (Fig. 5), indicating that Par-4 needs to ablate the activity of the atypical PKCs to promote cell death.

The Down-regulation of Endogenous Par-4 by Oncogenic Ras Sensitizes NIH-3T3 Cells to TNFα-induced NF-κB Activation—We have recently shown that the expression of oncogenic Ras in NIH-3T3 cells completely down-regulates basal levels of endogenous Par-4 by reducing its mRNA content. This is a very interesting observation because it provides us with a useful model system to address the role of changes in endogenous Par-4 levels on NF-κB activation, under a determined pathophysiological condition such as the Ras-induced oncogenic transformation. Therefore, a control vector or the HA-tagged oncogenic Ras mutant, Ha-RasV12, either alone or together with Myc-tagged Par-4, were transfected into NIH-3T3 cells. Afterward, cells were either untreated or stimulated with 10 or 30 ng/ml TNFα for 30 min. Cells were analyzed by triple immunofluorescence microscopy with the monoclonal anti-HA antibody 12CA5 to detect the expressed oncogenic Ras, with a rabbit polyclonal anti-Myc antibody to detect the expressed Par-4, and a goat polyclonal anti-p65 antibody to detect the nuclear translocation of p65. Results of Fig. 6 show that the simple expression of oncogenic Ras gives a small although reproducible nuclear translocation of p65 even in the absence of TNFα. The presence of this cytokine synergistically cooperates with oncogenic Ras to produce that effect. This was not only seen in NIH-3T3 cells (Fig. 6) but also in other cell systems such as HeLa cells, where the synergism between Ras and TNFα was even more pronounced (data not shown). Because Ras transformation promotes the down-regulation of Par-4, together with the observations reported here that Par-4 expression inhibits NF-κB activation, these results can be interpreted as that the reduction of Par-4 levels promoted by oncogenic Ras sensitizes cells to respond to TNFα-induced NF-κB. Consistent with this model, the ectopic expression of Par-4, which cannot be down-regulated by oncogenic Ras, inhibits the effects of Ras on p65 nuclear translocation (Fig. 6). Collectively, these observations are in good agreement with the notion that changes in the levels of Par-4, either ectopic or endogenous, influence NF-κB signaling.

DISCUSSION

The molecules involved in signaling events controlling cell death and survival are important targets for novel therapies in cancer and inflammatory diseases. The recent discovery that the product of par-4, a gene induced in cells undergoing apoptosis (26), binds to and inhibits the atypical subfamily of PKCs (3, 43), suggests that these kinases play important roles in the control of cell survival. Actually, the overexpression of wild-type PKC or λ/PKC (3, 6, 43) protects cells from apoptosis induced by different genotoxic and stress insults. Therefore, the investigation of the mechanisms whereby the atypical PKCs regulate cell function is of potential great interest. In this regard, the ability of the atypical PKCs to regulate ERK (3, 46) seems important from the point of view of programmed cell death because the basal ERK activity has been shown to be critical for cell survival at least in some cell systems (22, 47). Therefore, one of the mechanisms whereby the inhibition of the atypical PKCs by Par-4 induces apoptosis could at least in part involve the reduction of the ERK activity (3, 46). This pathway is antagonized by serum survival factors acting through the phosphatidylinositol 3-kinase/Akt cascade (46), unveiling an interesting cross-talk between different signaling events, which explains why Par-4 induces apoptosis more efficiently in cell cultures maintained under low serum conditions.

There are other apoptotic pathways that are activated in a more direct way such as those of the Fas system (46). Briefly, Fas binds to the adapter molecule FADD, which interacts with

2 M. Barradas, A. Monjas, M. T. Diaz-Meco, M. Serrano, and J. Moscat, submitted for publication.
caspase-8 (also known as FLICE/MACH) triggering the whole caspase cascade (46). Apoptosis induced by TNFα, although it shares some components of the Fas signaling cascade, is an interesting example of the activation by a single cytokine of two different pathways with opposite effects (30–32). Thus, TNFα triggers the caspase-8 route to apoptosis and simultaneously activates NF-kB through independent although highly interconnected elements (29). NF-kB is an important survival signal, and TNFα induces apoptosis if the NF-kB pathway is inactivated (30–32). Because the atypical PKCs contribute to the activation of NF-kB by regulating the IKK enzymatic activity, in the study reported here, we sought to investigate if Par-4 may provide a signal that inactivates NF-kB, allowing TNFα to induce apoptosis. We show in this study that expression of Par-4 blocks NF-kB and IKK activation in a manner that is dependent on the inhibition of the atypical PKCs. These are very interesting observations because they provide independent evidence, other than those obtained with dominant negative mutants (11, 13, 14, 17, 43), pseudosubstrate peptide inhibitors (15), or antisense oligonucleotides (16), that the atypical PKCs are critically involved in the control of NF-kB and IKK activation (41). Also of potential functional relevance is our finding that expression of Par-4, which by itself is a relatively weak inducer of apoptosis in the presence of high serum concentrations, allows TNFα to promote cell death, most probably by inhibiting NF-kB. Collectively, these results set an scenario in which the inhibition of the atypical PKCs by Par-4 induces apoptosis in resting cells by reducing ERK activity if the serum/phosphoinositide 3-kinase/Akt system is simultaneously inactivated (46). However, if the induction of apoptosis is an active process, as in the case of TNFα signaling, Par-4 contributes to cell death by inactivating the stimulation of the NF-kB survival branch. This suggests that potential inhibitors of the atypical PKCs could be used as sensitizers in cancer therapy. How NF-kB promotes cell survival is a matter of recent interest. Thus, Baldwin and co-workers (48) have demonstrated that the induction of TRAF1, TRAF2, cIAP1, and cIAP2 may account for the anti-apoptotic effects of TNFα signaling. In addition, other molecules such as IEX-1L (49) have also been demonstrated to mediate NF-kB-induced protection. Future work in our laboratory will address the impact that Par-4 and the atypical PKCs have on the expression of these survival genes.

A critical question that arises from this study is the pathophysiologic implications of these findings. We have evidence that tumor transformation by the Ras oncogene provokes a profound depletion of Par-4 mRNA and protein levels (see above). This may be of potential functional relevance from the point of view of tumor progression because it may constitute a mechanism whereby cancer cells are able to survive and proliferate more efficiently than the normal cells. We demonstrate here that the down-regulation of Par-4 by oncogenic Ras has a measurable impact on TNFα signaling toward NF-kB activation. Thus, cells expressing oncogenic Ras display a much more efficient nuclear translocation of p65 in response to TNFα than control cells. This also seems to be dependent on the down-regulation of Par-4, because its ectopic expression dramatically inhibits Ras synergistic actions on NF-kB. Together, all these results will be in good agreement with a role of Par-4 during tumorigenic transformation. In this regard, recent data from Rangnekar and co-workers (50) demonstrate the complete depletion of Par-4 in renal cell carcinoma. Of potential interest, these authors presented evidence that the ectopic expression of Par-4 in cell lines derived from those tumors sensitizes them to TNFα-induced apoptosis. This is clearly in keeping with the data reported in this paper and with the observations that down-regulation of NF-kB severely impairs Ras-induced transformation (51). Therefore, Par-4 emerges a novel tumor suppressor gene acting on the aPKCs-IKK signaling axis.
(1996) Mol. Cell. Biol. 16, 105–114
44. Johnstone, R. W., See, R. H., Sells, S. F., Wang, J., Muthukkumar, S., Englert, C., Haber, D. A., Licht, J. D., Sugrue, S. P., Roberts, T., Rangnekar, V. M., and Shi, Y. (1996) Mol. Cell. Biol. 16, 6945–6956
45. Sanchez, P., De Carcer, G., Sandoval, I. V., Mascat, J., and Diaz-Meco, M. T. (1998) Mol. Cell. Biol. 18, 3069–80
46. Berra, E., Diaz-Meco, M. T., and Moscat, J. (1998) J. Biol. Chem. 273, 10792–10797
47. Canman, C. E., and Kastan, M. B. (1996) Nature. 384, 213–214
48. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) Science 281, 1680–1683
49. Wu, M. X., An, Z., Prasad, K. V., Wu, R., and Schlossman, S. F. (1998) Science 281, 998–1001
50. Cook, J., Krishnan, S., Ananth, S., Sells, S. F., Shi, Y., Walther, M. M., Linehan, W. M., Sukhatme, V. P., Weinstein, M. H., and Rangnekar, V. M. (1999) Oncogene 18(5), 1205–1208
51. Mayo, M. W., Wang, C. Y., Cogswell, P. C., Rogers-Graham, K. S., Lowe, S. W., Der, C. J., and Baldwin, A. S., Jr. (1997) Science 278, 1812–1815
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