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The DNA-Dependent Protein Kinase Participates in the Activation of NFκB Following DNA Damage

Subimal Basu, Kenneth R. Rosenzweig, Matthew Youmell, and Brendan D. Price[1]
Joint Center for Radiation Therapy, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115

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The NFkB transcription factor is activated by diverse stimuli, including Ionizing Radiation (IR) and the cytokine TNFα. The role of DNA-PK, a protein kinase involved in the response to DNA damage, in the activation of NFκB by IR and TNFα was examined. In M059K cells, which express DNA-PK, NFκB was activated by both TNFα and IR. In M059J cells, which do not express DNA-PK, IR did not activate NFκB, whereas TNFα induction of NFκB was still observed. In HeLa cells, wortmannin, an inhibitor of DNA-PK, blocked the induction of NFκB by IR but not by TNFα. DNA-PK also phosphorylated the NFκB inhibitory proteins IkB-α and IkB-β *in vitro,* and deletion analysis demonstrated that DNA-PK phosphorylates 2 distinct regions of IkB-β. These results indicate that DNA-PK participates in the activation of NFκB by IR but not by TNFα. © 1998 Academic Press

The Rel/NFκB family of transcription factors play a key role in the cellular response to DNA damage [1-3]. NFκB is activated by UV and X-ray irradiation, cytokines and free radicals [4-7]. Inhibition of NFκB activation results in increased apoptosis following exposure to either TNFα or DNA damaging agents [1-3]. This suggests that activation of NFκB by various agents, including DNA damage, may suppress the apoptotic response.

NFκB is maintained in an inactive state in the cytoplasm by complexing with members of the IkB inhibitory protein family, including IkB-α [4,8] and IkB-β [9]. This interaction with IkB-α masks NFκB's nuclear localization signal and inhibits the DNA binding activity of NFκB [4]. The phosphorylation of serines 32 and 36 of IkB-α [8] by the recently cloned IkB-α kinase [10-12] stimulates the ubiquitination of IkB-α, followed by degradation of IkB-α by the 26S proteasome complex [8]. NFκB is then translocated to the nucleus and activates transcription of a variety of genes. IkB-β also participates in the activation of NFκB. Hypophosphorylated IkB-β binds to NFκB, but this interaction does not block the DNA binding or transcriptional activities of NFκB. Instead, hypophosphorylated IkB-β prevents the binding of IkB-α to NFκB [13,14]. The phosphorylation of the C-terminal of IkB-β by Casein Kinase II converts IkB-β to a form which inhibits the DNA binding activity of NFκB [14,15]. IkB-β therefore appears to regulate both the activation and repression of NFκB depending on the level of IkB-β phosphorylation.

The signal transduction pathway which links X-ray induced DNA damage to the activation of NFκB has not been identified. In this study, we have examined the role of the DNA-dependent Protein Kinase (DNA-PK) in the activation of NFκB by DNA damage. DNA-PK is a serine/threonine kinase consisting of an 86kd regulatory protein and a 460kd catalytic domain [17]. The 86kd regulatory protein binds specifically to the ends of double-stranded DNA, causing activation of DNA-PK's kinase activity [18]. Cells which are deficient in DNA-PK activity fail to repair double-strand DNA breaks, have faulty V(D)J recombination and are extremely sensitive to radiation [17]. DNA-PK therefore plays a central role in detecting DNA strand breaks and in regulating the cellular response to DNA damage. Here, we show that DNA-PK phosphorylates IkB-α and IkB-β, and provide evidence that functional DNA-PK is required for the activation of NFκB following exposure to DNA damaging agents, but not following exposure to TNFα.

MATERIALS AND METHODS

Cells. Cells were maintained in Hams-F12 (HeLa cells) or DMEM/Hams-F12 (M059J and M059K cells) with 10% Fetal Bovine Serum. Reagents were obtained from: p50 and p65 antibodies (Santa Cruz Biotech, CA); Wortmannin, TPA and etoposide (Sigma, St. Louis, MI); TNFα (Boehringer-Mannheim, IN).

[1] Corresponding author: B.D. Price, JF209, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Fax: (617) 632-4779. E-mail: brendan_price@dfci.harvard.edu.

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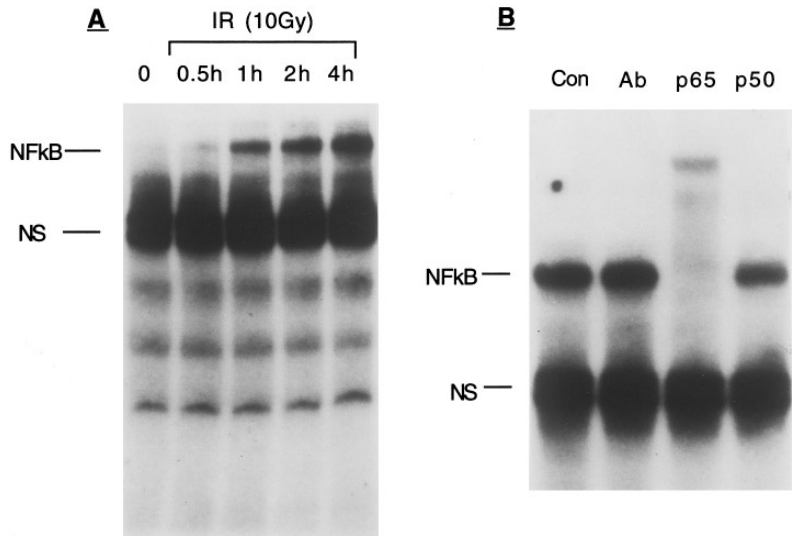

FIG. 1. Activation of NFκB by IR. A. HeLa cells were mock irradiated (0) or irradiated (10Gy) and NFκB DNA binding activity was measured at the indicated times. B. Cell Extracts from irradiated HeLa cells were incubated for 15min with either buffer (Con), non-specific antibody (Ab; 1μg), anti-p65 antibody (p65; 1μg) or anti-p50 antibody (p50; 1μg) prior to analysis by EMSA. ns, non-specific bands.

Electrophoretic gel mobility shift assays (EMSA). Cell lysates were prepared as described [19]. EMSA reactions contained: Cell lysate (10μg), [^{32}P]-NFκB consensus oligonucleotide (AGTTGAGGGGACTTTCCCAGGC: 0.5ng), BSA (20μg), pdI-dC (2μg), Buffer D+ (2μl), Buffer F (4μl) and DTT (1mM) in 20μl. Buffer D+: [20mM Hepes pH 7.9, 20% glycerol, 100mM KCl, 0.5mM EDTA, 0.25% NP40]. Buffer F: [20% FICOLL 400, 100mM Hepes pH7.9, 300mM KCl].

Fusion proteins and kinase assays. pGEX-2T-IkB-β was constructed by inserting a BamH1/EcoR1 fragment of IkB-β into pGEX-2T. Fusion proteins were purified as previously described [20]. IkB-β deletions were generated by PCR and inserted into pGEX-2T using the primers: **M:** CGCGGATCCATGGCCGGGGTCGCGTGCTTGG. **N:** CGCGGATCCATGCCCGGAGGACCAGGTCTG. **G:** CCGGAATTCTCAAGGGCTAAGCTTATG. **F:** CCGGAATTCTGAATCATATTCATCGCCCTCATCTCTGTT. **E:** CCGGAATTCTCAGGCAGGGTTGGGGTCATCAGG.

DNA-PK kinase assays contained 0.5Sp1 units of purified DNA-PK protein (Promega, Madison WI) in 10μl of kinase buffer [25mM Hepes, pH7.5; 50mM KCl; 10mM $MgCl_2$; 20% v/v glycerol; 0.1% NP-40; 20μM ZnCl; 1mM dithiothreitol]. 10μl of reaction mix [0.5μg fusion protein; 0.2μg calf thymus DNA; 10μM ATP; 10μCi [^{32}P]-ATP; 13mM spermidine; 4mM $MgCl_2$] was added and incubated for 20 min at 25°C. Reaction products were separated by SDS-PAGE and phosphorylated proteins identified by autoradiography.

RESULTS

In fig 1, the ability of Ionizing Radiation (IR) to activate NFκB in HeLa cells was examined. NFκB DNA binding activity was measured by EMSA using the consensus NFκB DNA binding site. IR induced the appearance of a slowly-migrating protein-DNA complex (fig 1A). This IR induced complex was supershifted by antibodies against the p65 sub-unit of NFκB (fig 1B) and binding was decreased in the presence of antibodies against the p50 subunit of NFκB (fig 1B: p50) but not by a non-specific antibody (fig 1B: Ab). The lower, non-specific bands (fig 1, ns) were unaffected by addition of either anti-p65 or anti-p50 antibodies. The IR induced band therefore represents an activated NFκB complex. The activation of NFκB in HeLa cells by IR was relatively slow, with maximal activation seen after 2-4h. Figure 1 demonstrates that a specific NFκB complex is activated in HeLa cells following exposure to IR.

Many cellular responses to DNA damage are mediated by members of the PI 3-Kinase gene family [22], which includes the Ataxia Telangiectasia (ATM) gene product [23] and the DNA-dependent protein kinase [DNA-PK; 22,26]. Recently, we demonstrated that the PI 3-kinase inhibitor wortmannin is a potent inhibitor of DNA-PK [24]. To determine if DNA-PK participates in the activation of NFκB by IR, HeLa cells were incubated with increasing concentrations of wortmannin and then exposed to IR. Wortmannin inhibited the IR induced activation of NFκB at 5μM (fig 2), which is similar to the concentration required to inhibit DNA-PK [24]. We also examined the ability of etoposide, a topoisomerase II inhibitor which generates strand breaks in DNA [21], to activate NFκB. Etoposide activated NFκB, and this activation was efficiently suppressed by wortmannin (fig 2). In fig 2, we also examined the ability of wortmannin to inhibit the activation of NFκB by non-DNA damaging agents such as TNFα and the phorbol ester TPA. The activation of NFκB by either TNFα or TPA was unaffected by wortmannin (fig 2). This indicates that a wortmannin sensitive step is required for the activation of NFκB by the genotoxic agents IR and etoposide, but not for activation by TNFα and TPA. Although wortmannin inhibits DNA-PK *in vivo* [24] wortmannin may inhibit other members of the PIK family, such as ATM, as well as other unidentified kinases [22-24]. To further examine the role of DNA-

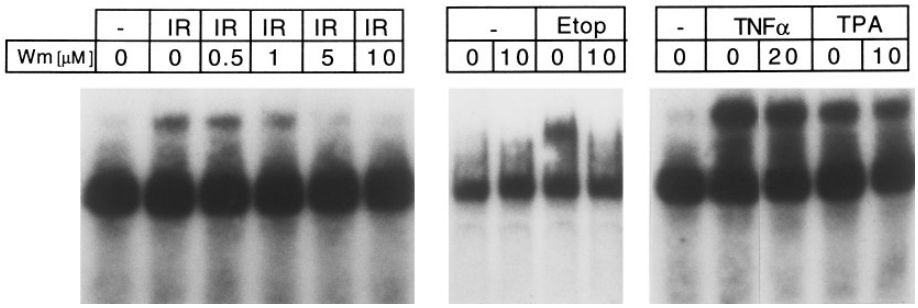

FIG. 2. Effect of wortmannin on the activation of NFκB. HeLa cells were preincubated for 15min in Wortmannin (Wm), and either irradiated (IR, 10Gy) or exposed to Etoposide (Etop, 50μM), TNFα (TNF, 10ng/ml) or TPA (TPA, 400nM). Cell extracts were prepared after 1h (TNFα & TPA) or 2h (IR & Etop) and analyzed by EMSA.

PK in the activation of NFκB by IR, we utilized cells with a defined genetic defect in DNA-PK activity. MO59J and M059K cells were isolated from distinct parts of a single human glioma biopsy [25]. M059J cells do not express detectable DNA-PK mRNA or protein, and are 30-fold more sensitive to radiation than the M059K cells [25]. M059J and M059K cells thus provide a system with a similar genetic background and defined DNA-PK status to examine the role of DNA-PK in the activation of NFκB by radiation. M059J and M059K cells were exposed to either IR or TNFα (fig 3), and the levels of NFκB activation assessed by EMSA. Exposure of both M059J and M059K cells to TNFα led to the appearance of a new protein-DNA complex (figure 3), which was super-shifted by the addition of anti-p65 antibodies, but not by non-specific antibodies. This indicates the presence of a TNFα activated NFκB protein complex in both the M059J and M059K cell lines. However, when the cells were irradiated, only the DNA-PK positive M059K cells displayed detectable activation of NFκB. No activation was seen in the DNA-PK negative M059J cells (fig 3). This result is consistent with the observation in fig 2 that the DNA-PK inhibitor wortmannin inhibits DNA damage induced activation of NFκB but not TNFα activation, supporting the hypothesis that activation of NFκB by IR involves DNA-PK.

The activation of NFκB occurs by phosphorylation of IkB-α followed by ubiquitin dependent degradation [8]. To determine if DNA-PK directly regulates the IkB proteins, we examined if IkB-α and IkB-β were substrates for phosphorylation by purified HeLa cell DNA-PK. Glutathione-S-Transferase (GST) IkB fusion proteins were incubated with purified HeLa cell DNA-PK (fig 4a). Since DNA-PK requires double-stranded DNA (dsDNA) for full activity [18], all DNA-PK assays were carried out in the presence and absence of dsDNA. GST protein was not phosphorylated by DNA-PK under any condition (fig 4a). Both GST-IkB-α and GST-IkB-β

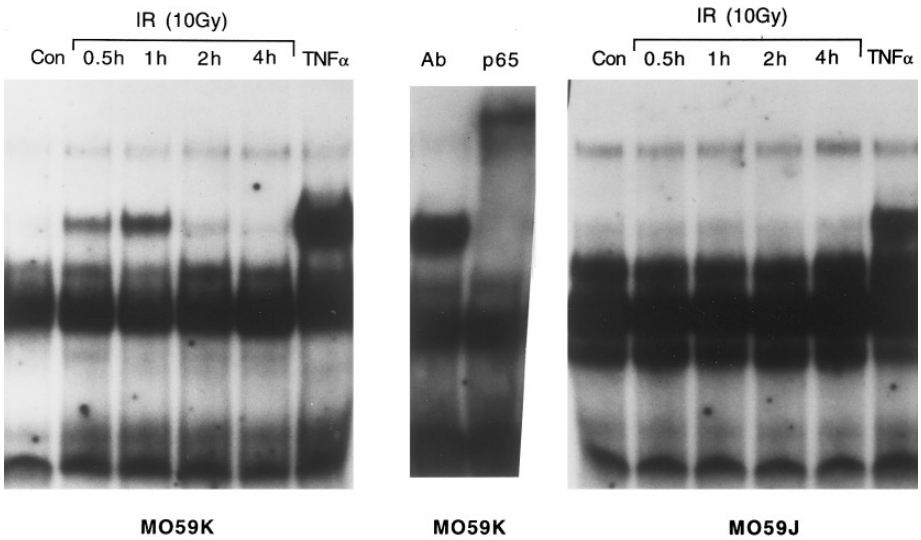

FIG. 3. NFκB is not activated by DNA damage in MO59J cells. M059K or M059J cells were stimulated with TNFα (TNF; 10ng/ml) for 30min or exposed to IR (10Gy) and the reactions terminated at the indicated time. EMSA was carried out as described in methods. TNFα stimulated extracts from M059K cells were incubated with antibodies to p65 or a non-specific antibodies (Ab) as indicated.

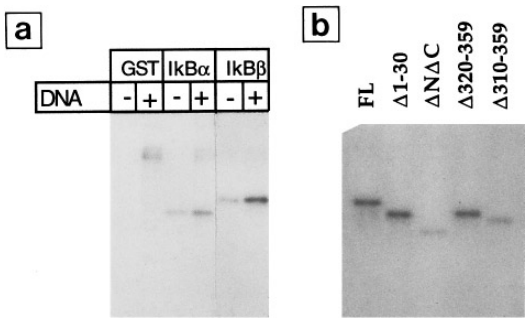

FIG. 4. Phosphorylation of IkB-α and IkB-β by DNA-PK. a. DNA-PK was incubated with GST, GST-IkB-α or GST-IkB-β in the absence (−) or presence (+) of calf thymus DNA to activate DNA-PK. b. DNA-PK was incubated with 0.5μg of Full Length GST-IkB-β (FL), or GST-IkB-β with deletions of amino-acids 1-30 (Δ1-30), amino-acids 320-359 (Δ320-359), amino-acids 310-359 (Δ310-359) or IkB-β spanning only amino-acids 30-310 ($\Delta N\Delta C$). All kinase reactions were carried out in the presence of calf thymus DNA.

were weakly phosphorylated by DNA-PK in the absence of exogenous DNA, and phosphorylation of both proteins was stimulated by addition of calf thymus DNA to activate DNA-PK. DNA-PK consistently phosphorylated IkB-β to a much greater extent than IkB-α. IkB-α incorporated 0.2±0.1 moles phosphate/mole protein, whereas IkB-β incorporated 1.5±0.2 moles of phosphate/mole of protein. This indicates that IkB-β is a better substrate for DNA-PK than IkB-α.

To determine the regions of IkB-β which were phosphorylated by DNA-PK, four deletions of IkB-β were generated by PCR and then analyzed for their ability to be phosphorylated by DNA-PK. In (Δ1-30)-IkB-β, the first 30 amino-acids from the N-terminal were deleted. This region contains two serines at positions 19 and 23 which are analogous to serines 32 and 36 in IkB-α which are responsible for inducible degradation of IkB-α [8,9]. The C-terminal (amino-acids 311-359) of IkB-β contains multiple serine and threonine residues, with the majority of serines located between amino-acids 310-320 within the sequence Cys-Ser-Ser-Ser-Gly-Ser-Asp-Ser-Asp-Ser [9]. Two C-terminal deletions were generated, Δ320-359, which still contains the serine rich region between amino-acids 310 and 320, and Δ310-359, with this region deleted. In addition, the central portion of IkB-β, lacking both the N- and C-terminal regions ($\Delta N\Delta C$, covering amino-acids 30-310), was examined. Both full length IkB-β, and IkB-β with a deletion of amino-acids 1-30 were efficiently phosphorylated by DNA-PK (fig 4b; Δ1-30). Deletion of amino-acids 320-359 from the C-terminal also did not alter the phosphorylation of IkB-β by DNA-PK (fig 4b; Δ320-359). However, deletion of amino-acids 310-359 (fig 4b; Δ310-359) caused a marked reduction in IkB-β phosphorylation by DNA-PK, although some phosphorylation of IkB-β by DNA-PK was still detected. When both the N- and C-terminal sequences were deleted, generating a protein containing only amino-acids 30-310, significant phosphorylation of IkB-β by DNA-PK was still detected (fig 4b; $\Delta N\Delta C$). The results indicate that there are at least two distinct regions of IkB-β phosphorylated by DNA-PK *in vitro*, and that these are located between amino-acids 30-310 and 310-320.

DISCUSSION

The results indicate that activation of NFκB by TNFα and TPA does not require DNA-PK, whereas NFκB activation by the DNA damaging agents IR and etoposide involves the participation of DNA-PK. This is supported by two separate lines of evidence. First, wortmannin inhibits activation of NFκB by both IR and etoposide, but not by TPA and TNFα. We have previously shown that wortmannin inhibits DNA-PK activity [24]. However, ATM and DNA-PK have significant homology within their kinase domain, suggesting that ATM, and other related proteins, may also be inhibited by wortmannin. To determine if DNA-PK plays a critical role in the activation of NFκB by IR, we examined M059J and M059K cells, which were isolated from a single human glioma biopsy and share a similar genetic background. M059J cells lack detectable DNA-PK activity [25], and failed to activate NFκB following exposure to radiation (fig 3). However, M059J cells still displayed NFκB activation following stimulation by TNFα, indicating the presence of activated NFκB in M059J cells. Although M059J cells may have other defects which contribute to the lack of IR induction of NFκB, it is more likely that the failure to see IR induced NFκB stems from the absence of DNA-PK. This combination of pharmacological studies and the DNA-PK defective cells indicates a key role for DNA-PK in the signal transduction pathway linking DNA damage to the activation of NFκB.

To further define the role of DNA-PK in the activation of NFκB, we examined if DNA-PK phosphorylates IkB-α and IkB-β. IkB-α was a poor substrate, whereas IkB-β was strongly phosphorylated by DNA-PK in two distinct regions located between amino-acids 30-309, and 310-320. Casein Kinase II (CKII) phosphorylates IkB-β at serines 313 and 315 [16], which is the same region phosphorylated by DNA-PK (fig 4). *In vitro* studies demonstrated that hypophosphorylated IkB-β binds to NFκB and displaces IkB-α, but does not block nuclear localization or DNA-binding of the NFκB complex [14]. The phosphorylation of IkB-β at serine 313 and 315 by Casein Kinase II converts IkB-β into a form which suppresses NFκB DNA binding activity *in vitro* [15,16] DNA-PK phosphorylates the same region of IkB-β as Casein Kinase II (amino-acids 310-320, fig 4). However, although we could demonstrate that IkB-β phosphorylated by Casein Kinase II inhibited NFκB DNA binding activity, IkB-β phosphorylated by DNA-

PK had no effect on NFκB DNA binding activity[2]. This difference may reflect the ability of DNA-PK to phosphorylate two distinct regions of IkB-β, including the central region containing the ankyrin repeat sequence, as well as the C-terminal Casein Kinase II region.

The exact function of the phosphorylation of IkB-β by DNA-PK in the DNA damage induced activation of NFκB is unclear. NFκB exists as an inactive NFκB-IkB complex in the cytoplasm. DNA-PK is mainly nuclear in HeLa cells, although a significant fraction of the DNA-PK protein is located in cytoplasm [26,27]. DNA-PK is thought to be activated by interaction with DNA strand breaks in the nucleus [18], making it unlikely that nuclear DNA-PK and cytoplasmic NFκB could directly interact. DNA-PK can translocate to the cytoplasm following stimulation of cells with EGF [27], but we have not seen translocation of DNA-PK following exposure to DNA damaging agents[2]. In HeLa cells, p50, IkB-α and IkB-β can be detected in the nucleus of both unstimulated and stimulated cells [15,28], suggesting that nuclear activation of NFκB complexes by DNA-PK occurs following DNA damage. However, the amounts of IkB and p50 in the nucleus are small [15,28], and may not be sufficient to account for the activation of NFκB seen after DNA damage. This suggests that other signaling intermediates are required for the activation of NFκB by DNA-PK. The ATM protein, which is also involved in regulating the DNA damage response, phosphorylates the C-terminal of IkB-α, and cells lacking ATM have deregulated NFκB expression [29]. ATM and DNA-PK may therefore cooperate in the activation of NFκB by genotoxic agents. DNA-PK also associates with c-abl and activates c-abl's kinase activity following exposure to DNA damage [23]. This interaction may also participate in the signaling required for the activation of cytoplasmic NFκB by DNA damage. The activation of NFκB by DNA-PK following DNA damage may therefore proceed through both direct phosphorylation of IkB-β by DNA-PK as well as through up-regulation of other DNA damage dependent signaling pathways.

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[2] S. Basu and B.D. Price, unpublished observation.

REFERENCES

1. Wang, C-Y., Mayo, M. W., and Baldwin, A. S. (1996) *Science* **274,** 784–787.
2. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784.
3. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789.
4. Miyamato, S., and Verma, I. M. (1995) *Adv. Cancer Res.* **66,** 255–292.
5. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20.
6. Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichselbaum, R., and Kufe, D. (1991) *J. Clin. Invest.* **88,** 691–695.
7. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258.
8. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeurle, P. A. (1995) *EMBO J.* **14,** 2876–2883.
9. Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) *Cell* **80,** 573–582.
10. Woronciz, J. D., Gao, X., Cao, M., Rothe, D., and Goeddel, V. (1997) *Science* **278,** 866–869.
11. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554.
12. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbossa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866.
13. Tran, K., Merika, M., and Thanos, D. (1997) *Mol. Cell Biol.* **17,** 5386–5399.
14. Suyang, H., Phillips, R., Douglas, I., and Ghosh, S. (1996) *Mol. Cell Biol.* **10,** 5444–5449.
15. Chu, Z-L., McKinsey, T. A., Liu, L., Qi, X., and Ballard, D. (1996) *Mol. Cell Biol.* **16,** 5974–5984.
16. McElhinney, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996) *Mol. Cell Biol.* **16,** 899–906.
17. Jackson, S. P., and Jeggo, P. A. (1994) *Trends Bio. Sci.* **20,** 412–415.
18. Gottleib, T. M., and Jackson, S. P. (1993) *Cell* **72,** 131–142.
19. Pahl, H. L., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2580–2588.
20. Price, B. D., Hughes-Davis, L., and Park, S. J. (1995) *Oncogene* **11,** 73–80.
21. Tishler, R. B., Calderwood, S. K., Coleman, C. N., and Price, B. D. (1993) *Cancer Res.* **53,** 2212–2216.
22. Hunter, T. (1995) *Cell* **83,** 1–4.
23. Kharbanda, S., Pandey, P., Jin, S., Inoue, S., Bharti, A., Yuan, Z-M., Weichselbaum, R., Weaver, D., and Kufe, D. (1997) *Nature* **386,** 732–735.
24. Rosenzweig, K. E., Youmell, M. B., Palayoor, S. T., and Price, B. D. (1997) *Clin. Cancer Res.* **3,** 1149–1156.
25. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S., Barron, G. M., and Allalunis-Turner, J. (1995) *Science* **267,** 1183–1185.
26. Carter, T., Vancurova, I., Sun, I., Lou, W., and DeLeon, S. (1990) *Mol. Cell Biol.* **10,** 6460–6471.
27. Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J., and Kumar, R. (1998) *J. Biol. Chem.* **273,** 1568–1573.
28. Read, M. A., Neish, A. S., Gerritsen, M. E., and Collins, T. (1996) *J. Immunol.* **157,** 3472–3479.
29. Jung, M., Kondratyev, A., Lee, S. A., Dimtchev, A., and Dritschilo, A. (1997) *Cancer Res.* **57,** 24–29.