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Hepatitis B Virus pX Activates NF-κB-Dependent Transcription through a Raf-Independent Pathway

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In this study, we characterized the molecular events involved in the activation of the ubiquitous transcription factor NF-κB by the viral transactivator pX. pX expression in HeLa cells determines a manyfold increase in NF-κB-dependent transcription, which is associated with an increase in p50/p65 heterodimer DNA-binding activity. Since the IκB-α inhibitory subunit proteolytic degradation, which follows its phosphorylation/modification, is a key event in NF-κB activation by different stimuli (such as growth factors, phorbol esters, tumor necrosis factor, UV irradiation, and oxygen radicals), we investigated pX effects on IκB-α, as well as the possible involvement of known signalling pathways in pX-induced NF-κB-dependent transcription. We observed that although pX had no direct effect on p50 or p65, it was able to restore the IκB-α-suppressed p50/p65 activity. More directly, the stable expression of pX in HeLa cells resulted in reduced levels of IκB-α in the cytoplasm. Pretreatment of the cells with H7, calphostin C, tyrphostin 25, or *N*-acetylcysteine did not impair the effects of pX on NF-κB, thus ruling out the involvement of protein kinase C, tyrosine kinases, and oxygen radicals. Finally, while most of the known NF-κB-activating agents converge on Raf-1 protein kinase, when Raf-1 activity is blocked by overexpression of a dominant negative mutant, the effects of pX on NF-κB are not impaired. Thus, we suggest that although pX is able to activate the Ras/Raf-1-signalling pathway, it triggers NF-κB activation by an as yet unidentified Raf-1-independent pathway.

Persistent hepatitis B virus infection is a major risk factor in the development of hepatocellular carcinoma (5). However, since liver cancer occurs several years after the infection, the precise role played by this virus is unclear. A likely transforming viral gene is the X gene, which encodes a transcriptional transactivator of cellular and viral genes (2, 3, 11, 34, 47, 53, 54, 56). Indeed, the X protein is able to transform normal rodent cells (25, 48) and can cause hepatocellular carcinoma in certain strains of transgenic mice (28). Moreover, it has been shown that pX can induce cell cycle progression in quiescent fibroblasts (29).

The molecular mechanisms underlying pX effects on transcription, cellular proliferation, and transformation are only partially defined. pX has been shown to (i) directly interact with DNA-binding proteins involved in transcriptional regulation (10, 21, 38, 44, 55) and cell growth control (14); (ii) modulate intracellular signalling pathways (41), including the protein kinase C pathway (12, 27) and the Ras/Raf signalling pathway (7, 12, 42); and (iii) interact with cellular proteases and components of the proteasome complex (16, 34a, 50) and with a putative DNA repair enzyme (31). In this study, we focused on the effects of pX on NF-κB, a transcription factor known to regulate a large number of genes involved in cell activation and growth control and to be activated by different viral proteins with an oncogenic potential, such as the human T-cell leukemia virus type 1 (HTLV-1) Tax (1, 24, 30, 33, 36) and Epstein-Barr virus LMP-1 (20, 23).

NF-κB belongs to a highly conserved Rel-related protein family, whose members bind to an array of homologous decanucleotide sequences with different affinities (for reviews, see references 4 and 51). κB sites bind homo- and heterodimers made up of different members of the Rel family of proteins (p50 or NF-κB1, p52 or NF-κB2, c-*rel* or Rel, p65 or Rel-A, Rel-B and Dorsal). Of these, generally the p50/p65 heterodimer (commonly referred to as NF-κB) is the most abundant and ubiquitous. In uninduced cells, NF-κB-Rel complexes are retained in the cytoplasm because of the interaction with a second family of proteins with a regulatory/inhibitory function, including IκB-α, IκB-β, p105, and p100 (6, 51). p50/p50 or p52/p52 complexes—which bind κB sites with a high affinity but do not effectively activate transcription—do not bind efficiently to IκB-α: thus, they are not retained in the cytoplasm and, within the nucleus, are entirely regulated by the oncoprotein bcl-3. It has been proposed that bcl-3 dissociates inactive p50 homodimers from their cognate binding sites on DNA (18), thus allowing transactivating NF-κB complexes to attach (17). Moreover, other studies indicate that bcl-3 transactivates directly through κB motifs, via association with DNA-binding p50 (19) and p52 (8) homodimers. Several agents (growth factors, phorbol esters, tumor necrosis factor alpha (TNF-α), UV irradiation) activate intracellular signalling pathways that, through the Raf-1 protein kinase (13, 15), converge on IκB-α, leading to its phosphorylation and/or modification and subsequent degradation (22), thus allowing NF-κB–Rel complexes to enter the nucleus and activate transcription. In both HeLa and PLC/PRF/5 cells, pX, but not a frameshift mutant, pX(FS), activated κB site-directed transcription (Fig. 1A; data not shown). pX did not possess any activating potential toward the minimal thymidine kinase (tk) promoter (plasmid pBL2-CATdel [Fig. 1B]) in the same cells. Moreover, it was unable to activate NF-κB in undifferentiated NTera-2 cells (8), not expressing detectable amounts of NF-κB–Rel proteins (data not shown). The ability of pX to activate NF-κB tran-

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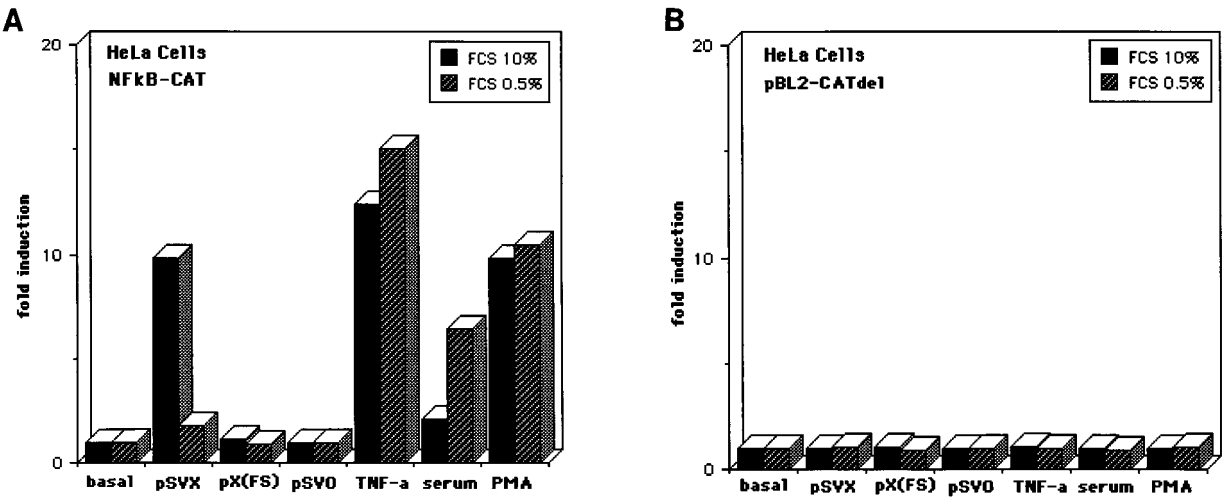

FIG. 1. pX activates NF-κB-directed transcription. (A) Human HeLa cells were plated at 30% confluency the day before transfection by the calcium phosphate coprecipitation technique, as described previously (14). Plasmids pSV-X and pSV-X(FS) (4 μg) express the wild-type or a frameshift mutant pX, respectively, under the control of the simian virus 40 promoter (34). Plasmid pSV0 (4 μg) contains only the simian virus 40 promoter/enhancer sequences (34). pNFκB-CAT (0.5 μg) contains the canonical NF-κB sites from the vimentin promoter cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (35). As the positive control for NF-κB activation, 1,000 UI of human recombinant TNF-α (Genzyme) per ml, 20% fetal calf serum, or 100 ng of phorbol myristate acetate (PMA; Sigma) per ml was used. The experiments were performed with both high-serum (10%) and low-serum (0.5%) culture medium. At 48 h after transfection, cells were collected and CAT assays were performed as described previously (41). (B) pX is unable to activate transcription from a minimal tk promoter. Plasmid pBL2-CATdel (1 μg) contains the CAT gene driven by the minimal tk promoter and differs from the original pBL2-CAT in that a 300-bp sequence containing a putative cyclic AMP response element upstream of the tk promoter has been removed (3, 42). Results are expressed as fold induction, which is the ratio of the percent conversion obtained in cotransfection experiment with the pX expression vector to the percent conversion obtained with the pUC19 plasmid. The results are the mean of results of at least three different experiments that differ by no more than 10%.

scriptional activity was greatly reduced when the cells were grown in low serum (0.5% fetal calf serum) (Fig. 1A). This suggests that for maximal pX activity, activation of signalling pathways by growth factors is required either to positively modulate the intrinsic pX activity or to sensitize NF-κB-activating pathways to the effects of pX (see also reference 12). pX expression also induced NF-κB DNA-binding activity (Fig. 2); by means of monoclonal antibodies directed toward different NF-κB–Rel proteins, we identified p50 and p65 as the major κB site-bound proteins in pX-expressing cells (Fig. 2).

To assess whether pX does affect the cytoplasmic pathway of NF-κB regulation or NF-κB DNA binding and/or the transcriptional activity of p50/p65 proteins, we carried out the following experiments. First, we observed that transfection and overexpression of p50, p65, or both provoke different degrees of activation of NF-κB-directed transcription, which is not further increased when pX is coexpressed (Fig. 3). Similar experiments performed with NTera-2 cells gave the same results (data not shown). This suggests that when p50/p65 heterodimers are expressed at a level that overcomes the inhibitory potential of endogenously expressed IκB proteins, pX is unable to upregulate their intrinsic transcriptional activity. The outcome of pX and p50 cotransfection is different, since IκB-α does not bind p50 homodimers (32). Indeed, exogenously transfected p50 probably homodimerizes, enters the nucleus freely, and finally binds κB sites, giving rise to a mild transcrip-

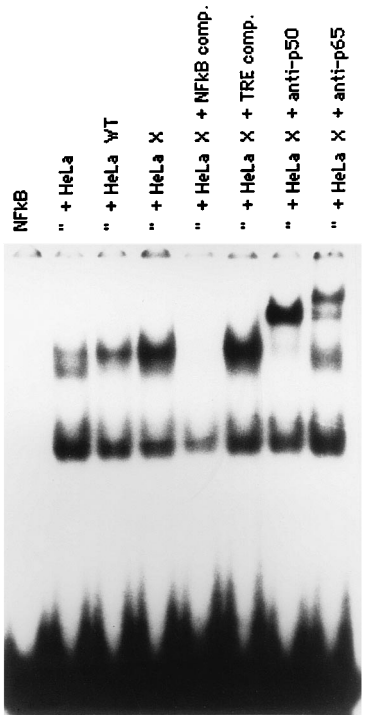

FIG. 2. Transient expression of pX induces DNA-binding activity of p50/p65 heterodimers. pX was expressed by using a recombinant vaccinia virus (a kind gift of F. Chisari) as described previously (41). Briefly, confluent cells were infected at 10 PFU per cell for 1 h. After this time, the cells were overlaid with fresh medium and harvested 16 h later. Nuclear extracts were prepared as described previously (41). A 6-μg portion of each extract was tested in an electrophoretic mobility shift assay with a ^{32}P-5′-end-radiolabeled oligonucleotide containing a canonical NF-κB site (5′AGTTGAGGGGACTTTCCCAG GC3′). The specificity of the retarded complexes was assessed by preincubating nuclear extracts with a cold NF-κB probe or an unrelated TRE probe. To determine the composition of the retarded complexes, 0.5 μg of rabbit polyclonal anti-p50 or anti-p65 antibodies (17) or of control antibodies (not shown) was incubated overnight with nuclear extracts from infected cells. The lower band appearing in the gel is a nonspecific complex, in that it is not inhibited by an excess of cold specific probe.

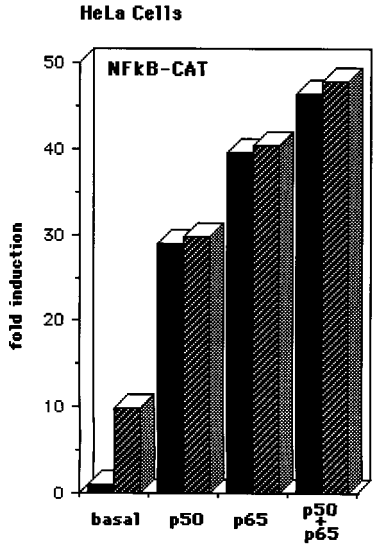

FIG. 3. pX is unable to directly activate p50 or p65. HeLa cells were cotransfected with pNFκB-CAT and with plasmids encoding p50 (0.5 μg), p65 (0.5 μg), and pX (4 μg). p50 and p65 were expressed from Rous sarcoma virus promoter-based expression vectors (8) and are kind gifts of U. Siebenlist. Results are expressed as fold induction (see above) and are the mean of results of three different experiments that differ by no more than 10%. Symbols: ■, - - - -; ▨, pX.

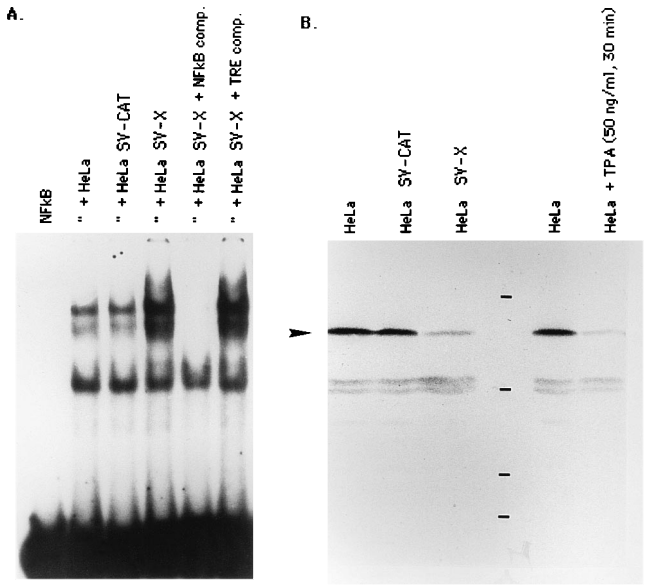

FIG. 5. (A) pX increases the NF-κB DNA-binding activity in stable transfectants. HeLa cells were transfected with 10 μg of plasmid pSV-X or pSV-CAT, together with 1 μg of plasmid pSV2-Hygro, which contains the hygromycin resistance gene under the control of the simian virus 40 early promoter. At 48 h after transfection, the cells were transferred to hygromycin-containing medium (200 μg of hygromycin per ml) and selected for 14 days. Clones were trypsinized and replated. Cell extracts were prepared from first- or second-passage cells. (B) IκB-α levels are markedly reduced in pX stable transfectants. Whole-cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 50 mM Tris (pH 7.5), and 1 mM phenylmethylsulfonyl fluoride. A 150-μg sample of each extract was run on a sodium dodecyl sulfate–12.5% acrylamide gel and blotted onto nitrocellulose (Schleicher & Schull). The filter was probed with an anti-IκB-α/MAD3 (c-15) rabbit polyclonal antibody (Santa Cruz) as specified by the manufacturer. As a positive control, IκB-α degradation by tetradecanoyl phorbol acetate is shown. The position of IκB-α in the gel is shown by an arrowhead.

tional activation, which cannot be further enhanced by pX. Second, the overexpression of IκB-α neutralizes whatever effect pX—as well as serum, TNF, phorbol esters, and H_2O_2—could have on NF-κB-directed transcription (Fig. 4A). On the other hand, pX is able to partially relieve the IκB-α-mediated suppression of p50/p65 transcriptional activity (Fig. 4B). The above experiments suggest that pX activates NF-κB by down-

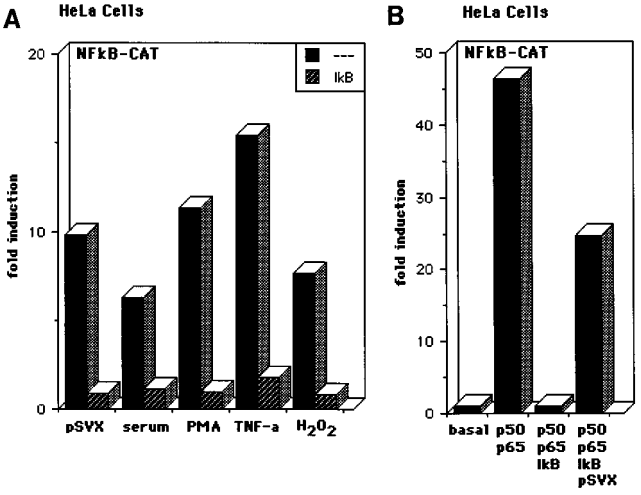

FIG. 4. (A) Overexpression of IκB-α blocks the effects of pX on NF-κB-directed transcription in HeLa cells. HeLa cells were transfected with pSV-X and pNFκB-CAT, with or without an expression vector encoding the human IκB-α under the control of the Rous sarcoma virus promoter (1 μg) (8) (a kind gift of U. Siebenlist). IκB-α overexpression also blocked the effects of phorbol myristate acetate (PMA), serum, TNF-α, and hydrogen peroxide on NF-κB. (B) pX expression interferes with the IκB-α-dependent suppression of p50/p65 transcriptional activity. p50 and p65 were expressed in HeLa cells together with IκB-α, with or without pX. Coexpression of pX mitigates IκB-α-dependent suppression of NF-κB-driven transcription.

regulating IκB-α activity. To assess this directly, we selected a polyclonal population of HeLa cells that stably express pX and evaluated the NF-κB/IκB status in the same cells. Indeed, we found that in pX transfectants, NF-κB DNA-binding activity is much higher than in mock-transfected control cells (Fig. 5A) and the levels of immunodetectable IκB-α are significantly down-regulated (Fig. 5B).

Thus, we can conclude that pX activates NF-κB by targeting the degradation of the inhibitory subunit, IκB-α. Similarly, both the Epstein-Barr virus LMP-1 protein and the HTLV-1 Tax protein induce IκB-α phosphorylation and degradation (23, 26, 49); in addition, Tax induces NF-κB nuclear translocation through the dissociation of cytoplasmic complexes containing the p105 and p100 precursors of p50 and p52 while being apparently unable to induce IκB-α degradation in pre-B cells (40). Interestingly, LMP-1 has recently been shown to interact with proteins involved in signal transduction from TNF receptor family cytoplasmic domains (39). Thus, it is very likely that the activation of NF-κB by LMP-1 reflects the propagation of signals similar to those activated by TNF receptor cross-linking.

Since the activation of NF-κB by TNF (and presumably by LMP-1), as well as by serum, oxygen radicals, and growth factors, occurs through a pathway inhibited by the functional blocking of Raf-1 and Ras (13, 15), we assessed the effects of dominant negative Ras and Raf mutants (Ras N17 and Raf C4, respectively) (9, 46) on NF-κB activation by pX. We found that

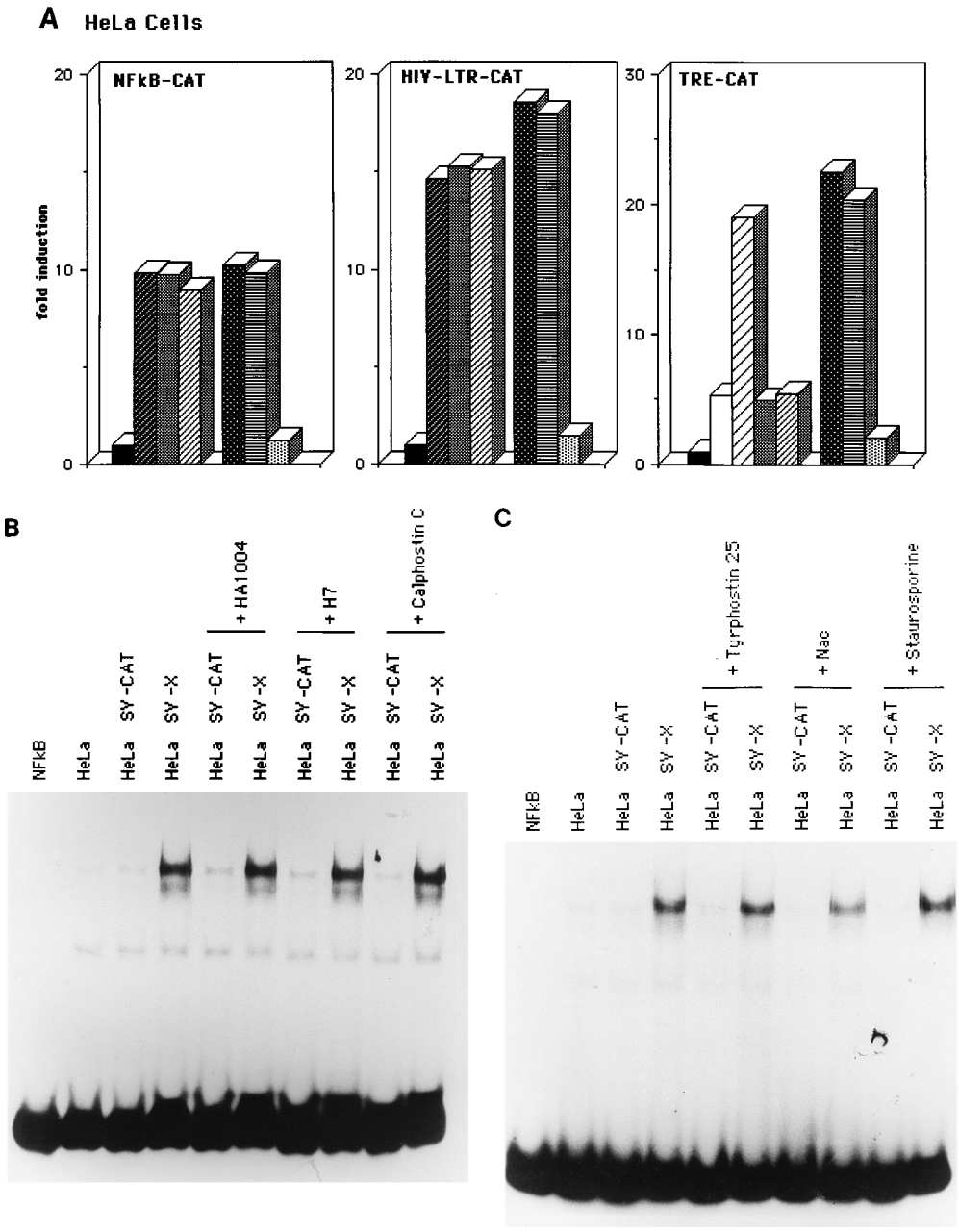

FIG. 6. (A) Activation of NF-κB by pX does not require Ras or Raf-1. HeLa cells were transfected with pNFκB-CAT (35), pSV-X (34), and vectors encoding either a dominant negative Ras mutant (pSV-Ras N17) (47) or a dominant negative Raf-1 mutant (pRSV-RafC4) (10) (4 μg each). The effects of constitutively active Ras and Raf mutants (Ras Val-12 and Raf BXB, respectively) (9, 47) on NF-κB-dependent transcription are shown (left panel). Similar results were obtained with, as a target for pX activity, the human immunodeficiency virus type 1 long terminal repeat (HIV-LTR) (central panel), whose activation by pX depends mainly on the NF-κB sites (4a, 51). The effects of a functional block in the Ras/Raf pathway on pX-dependent induction of c-Jun activity are also shown (right panel). Symbols: ■, basal; □, c-Jun; ▨, c-Jun plus pSVX; ▨, pSVX; ▩, pSVX plus Ras Asn-17; ▨, pSVX + Raf C4b; ▩, Ras Val-12; ▤, Raf BXB; ▨, Val-12 + C4b. (B) Effects of protein kinase C and A inhibitors on pX-induced activation of NF-κB. The protein kinase C inhibitors H7 and calphostin C were used at 0.1 mM and 1 μM, respectively (41). The protein kinase A inhibitor HA1004 was used at 75 μM (41). All inhibitors were added to the culture medium 5 h before the cells were harvested. (C) Effects of tyrosine kinases inhibitors, oxygen radical scavengers, and staurosporine on pX-mediated NF-κB activation. *N*-Acetyl-L-cysteine (Nac) was used at a final concentration of 30 mM; the final concentrations of tyrphostin and staurosporine were 20 μM and 50 ng/ml, respectively.

when pX was cotransfected with either Ras N17 or Raf C4 expression vectors, its ability to activate NF-κB was not impaired (Fig. 6A, left panel). On the other hand, the effects of pX on c-Jun transcriptional activity were almost completely inhibited by the expression of either dominant negative mutant (Fig. 6A, right panel) (42). The outcome of this experiment would suggest that while pX is able to activate the Ras/Raf signalling pathway in these cells (in spite of the role of this pathway in the activation of c-Jun/AP-1 by pX [7, 42]), it is not necessary for the activation of NF-κB.

Moreover, according to a previous report (37), the effects of pX on NF-κB were not blocked by chemical inhibitors of protein kinase C (calphostin C and H7) (Fig. 6B) and were not impaired by scavengers of oxygen radicals (*N*-acetylcysteine) or

by tyrosine kinase inhibitors or staurosporine (which is an almost general inhibitor of protein kinases) (Fig. 6C).

In summary, the results presented in this paper demonstrate that pX can activate NF-κB transcription factor through a "classical" mechanism, in that it involves the degradation of the inhibitory subunit IκB-α. However, unlike other known NF-κB inducers—such as TNF, phorbol esters, and interleukin-1—pX activates an IκB-α degradation pathway independent of the activity of Ras, Raf-1, and protein kinase C and of the generation of oxygen radicals. As regards the viral proteins able to target IκB-α degradation, it has been shown that Tax-induced NF-κB activation is blocked by antioxidants (45). Although a direct demonstration is still lacking, considering its ability to interact with components of the TNF receptor signalling pathway, the effect of LMP-1 on NF-κB is also likely to depend on the generation of oxygen radicals. It will be of particular interest to test whether Tax- and LMP-1-induced IκB-α degradation occurs through proteolysis (43, 52) and since pX has been shown to interact with cellular proteases, in particular, with a protein of the proteasome complex (16, 34a), it would be tempting to speculate that pX interferes directly in the activity of proteolytic enzymes involved in IκB-α degradation rather than influencing the cellular pathways leading to preproteolytic IκB-α modifications.

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