RAC-3 is a NF-κB coactivator

Santiago Werbajh[a,1], Ignacio Nojek[a,1], Rainer Lanz[b], Mónica A. Costas[a,2,*]

[a] *Laboratorio de Fisiología y Biología Molecular, Departamento de Cs. Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, C1428EHA Buenos Aires, Argentina*
[b] *Molecular and Cellular Biology, Baylor College of Medicine. Houston, TX 77030, USA*

Received 19 September 2000; revised 26 October 2000; accepted 28 October 2000

First published online 9 November 2000

Edited by Julio Celis

Abstract It has been shown that the molecular mechanism by which cytokines and glucocorticoids mutually antagonize their functions involves a mutual glucocorticoid receptor (GR)/nuclear factor-κB (NF-κB) transrepression. Here we report a role for the nuclear receptor coactivator RAC3, in modulating NF-κB transactivation. We found that RAC3 functions as a coactivator by binding to the active form of NF-κB and that overexpression of RAC3 restores GR-dependent transcription neglecting GR/NF-κB transrepression. The competition between GR and NF-κB for binding to RAC3 may represent a general mechanism by which both transcription factors mutually antagonize their activity.

Key words: Nuclear receptor coactivator; Nuclear factor-κB; Tumor necrosis factor; Glucocorticoid receptor

1. Introduction

Nuclear factor-κ-B (NF-κB) is a ubiquitous mammalian transcription factor that is activated in response to a wide variety of extracellular stimuli including endotoxins and inflammatory cytokines such as interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) [1–3]. In addition to its pivotal role in immune response and inflammation, NF-κB regulates the expression of genes that control cell cycle [4,5] and cell viability [6–10]. NF-κB consists of dimers of proteins containing the Rel dimerization domain, the p50/p65 (Rel-A) heterocomplex being the best characterized at present. Inactive NF-κB is trapped in the cytoplasm by association with IκB inhibitor proteins. Phosphorylation of IκB by specific kinases activated by extracellular signals mark IκB for degradation, thereby allowing activation of the NF-κB complex, which subsequently translocates to the nucleus to modulate target gene expression by binding, in a sequence-specific fashion, to κB promoter elements [1–3].

Inflammatory cytokines acting at the hypothalamic–pituitary–adrenal axis induce the synthesis of glucocorticoids, which function as major immunosuppressive agents in mammals [11–15]. Generally, glucocorticoids control gene expression by binding to and thereby activating the glucocorticoid receptor (GR), which subsequently translocates to the nucleus and binds to the glucocorticoid response element (GRE) at target gene promoters [16].

The molecular mechanism by which glucocorticoids exert immunosuppression has been believed to involve: (a) interference at the κB-responsive promoter due to a physical interaction between GR and Rel-A and (b) the induction of IκB expression which prevents NF-κB activation [17–19].

In the past few years it has become evident that controlled transcription involves the participation of coactivators, a growing family of different molecules with intrinsic transactivation domains and various additional enzymatic functions.

Some 'general' coactivators are: CBP (cyclic AMP response element binding protein (CREB)-binding protein) or its homolog p300, and molecules of the p160 coactivator family, which consists of SRC-1 (steroid receptor coactivator-1), TIF-2 (transcriptional intermediate factor-2), and RAC3 (mouse SRC-3). They participate in transcriptional regulation by interacting with a great number of nuclear receptors and various transcription factors [20–23].

SRC-1 has been shown to interact with NF-κB by binding to p50 thereby enhancing κB-dependent transcriptional activity [24]. Similarly, CBP binds to p65 and enhances NF-κB-mediated transactivation in a protein kinase A (PKA)-dependent manner [25]. In addition, Sheppard et al. demonstrated that NF-κB recruits a coactivator complex that has striking similarities to that recruited by nuclear receptors [26]. Overexpression of CBP/p300 or SRC-1 was shown to relieve mutual GR/NF-κB transrepression, indicating a possible competition for limiting amounts of coactivators for controlled κB transactivation [27]. In contrast to these observations, McKay and Cidlowski have recently reported that the mechanism of transrepression between both transcription factors is not so simple and they propose an integrative model, where CBP plays a critical role stabilizing the GR/NF-κB interaction and enhancing their antagonism [28]. Moreover, there is additional evidence supporting the hypothesis that glucocorticoids repress NF-κB activity by disturbing the Rel-A interaction with the basal transcription machinery, irrespective of the levels of expression of coactivators in the cell [29].

Although there is a growing source of evidence showing the complexity in the cross-talk between NF-κB and GR, it is not known whether other members of the p160 family play similar roles in response to physiological stimuli to activate NF-κB. In particular, RAC3, a gene overexpressed in primary breast tumor tissues [30], has not been reported to be involved in the regulation of κB transcription to date. Therefore, we decided to investigate the role of RAC3 on TNF-α-induced NF-κB transcriptional activity and its effect on the mutual GR/NF-κB transrepression. We found that RAC-3 is an NF-κB co-

*Corresponding author. Fax: (54)-11-4576 3321.
E-mail: mcostas@bg.fcen.uba.ar

[1] Both authors contributed equally to this work.
[2] Member of the Argentine National Research Council (CONICET).

0014-5793/00/$20.00
PII: S0014-5793(00)02223-7

activator, and that the overexpression of RAC3 overcomes the mutual GR/NF-κB transrepression.

2. Materials and methods

2.1. Cells and reagents

Human cervix carcinoma HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% charcoal-stripped steroid-free fetal calf serum (Gibco, Paisley, UK), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Unless stated, reagents were obtained from Sigma (St. Louis, MO, USA) or Pharmacia (Uppsala, Sweden).

2.2. Expression vectors and reporter plasmids

κB-Luc reporter plasmid was kindly provided by Dr. Omar Coso, Universidad de Buenos Aires, Argentina; ssIκB expression vector carrying mutated Ser 32 and Ser 36 to prevent phosphorylation and proteolysis of IκB was generously provided by Dr. G. Cadwell and Dr. M. Karin, University of California, La Jolla, CA, USA; and human Rel-A expression vector was provided by Dr. J. DiDonato, Cleveland Clinic. GRE reporter plasmid (MMTV-Luc) and expression vectors for coactivators pCR3.1-SRC-1 and pVL-CBP were previously described [31]; pSG5-TIF-2 was kindly provided by Dr. H. Gronemeyer [32] and pCMX-RAC3 was a gift from Dr. R. Evans, The Salk Institute, San Diego, USA.

2.3. Transfections assays

Cells were cultured in six-well plates at a density of 3×10^5 cells/well in DMEM without serum or antibiotics and transiently transfected with totally 3–4 μg of DNA (including 0.5 μg of reporter and 0.5 μg of constitutive RSV-β-gal control vector) using lipofectamine (Gibco BRL). After 5 h transfection medium was replaced by DMEM 10% charcoal-stripped serum. Cells were stimulated with human TNF-α (Calbiochem) at a concentration of 10 or 20 ng/ml and dexamethasone (DEX) at a concentration of 50 or 500 nM, as indicated, and cultured for 24 h prior harvesting.

Cellular extracts for Luc and β-gal assays were prepared, and assays were performed using the appropriate substrates following the manufacturer's protocols (Promega).

2.4. Immunoprecipitations and Western blots

Co-immunoprecipitations were performed as previously described [31]. Briefly, transfected HeLa cells overexpressing HA-tagged RAC3 or transfected with empty control vector were stimulated with 10 ng/ml of TNF-α for 45 min and then lyzed in a buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40) containing 1 mM dithiothreitol and the protease inhibitors 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride. Supernatants of lysates were incubated overnight at 4°C with HA antibody (Santa Cruz Biotechnology, CA, USA) and immunoprecipitated for 2 h at 4°C with GammaBind G Sepharose (Pharmacia Biotech, USA). After six washes, Sepharose-bound immunocomplexes were separated on 8% SDS–PAGE and electro-transferred to nitrocellulose membranes. Membranes were blocked for non-specific binding with a solution containing 5% bovine serum albumin (IgG-free) and 0.05% Tween-20 and incubated for 60 min at room temperature in phosphate-buffered saline and 0.5 μg/ml Rel-A antibody (Santa Cruz Biotechnology, CA, USA). Subsequently, membranes were incubated for 45 min with horseradish peroxidase-conjugated secondary antibody, and the proteins visualized by autoradiography using the chemiluminescence luminol reagent (Santa Cruz Biotechnology, CA, USA).

3. Results and discussion

3.1. Effect of RAC3 on NF-κB transcriptional activity induced by TNF-α

In order to determine the role of RAC3 on NF-κB transcriptional activity, we transiently transfected HeLa cells with different amounts of RAC3 expression vector along with κB-Luc reporter construct and stimulated NF-κB transactivation by treating the cells with TNF-α. As shown in Fig. 1, overexpression of RAC3 significantly enhanced TNF-α-induced basal NF-κB transcriptional activity in a dose-dependent manner. Similar results were obtained with cells that were transiently overexpressing Rel-A in order to emulate constitutive NF-κB transactivation (data not shown). These results demonstrate that RAC3 enhances NF-κB-transcriptional activity in response to physiological stimuli such as TNF-α.

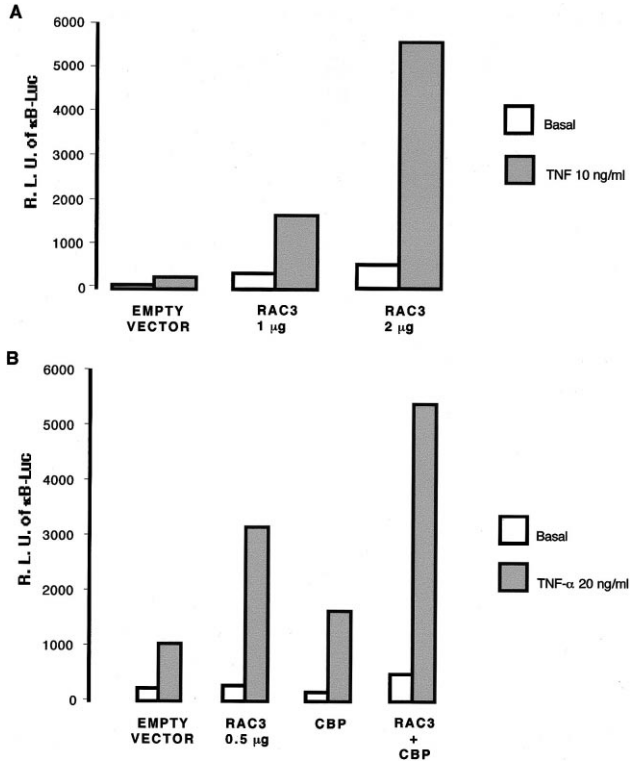

Fig. 1. Role of RAC3 on κB-mediated transcription. HeLa cells were transfected with 500 ng κB-Luc reporter plasmid, 500 ng of RSV-β-GAL and 2 μg of either pCMX empty vector or pCMX-RAC3 (A) or 500 ng pVL-CBP (B) and the cells stimulated with TNF-α as indicated. Bars represent the average of relative light units (RLU) of assay. Similar results were obtained in three independent experiments, S.D. was less than 10%.

Due to its ability to interact with other coactivators, CBP has been identified as a crucial component for nuclear receptor transactivation [20–23]. In addition, it was previously shown that CBP binds physically to the p50 component of NF-κB [25]. In agreement with these observations, we found that overexpression of CBP also enhanced NF-κB transcription, albeit to a lesser extent than the coactivation by RAC3. The overexpression of both coactivators resulted in an additive transcriptional coactivation (Fig. 1B).

Previous studies have shown that cells transiently transfected with Rel-A require TIF-2 or SRC-1 for κB-dependent transcription [26]. Accordingly, we report here that both coactivators enhanced NF-κB transcriptional activity in HeLa cells stimulated by TNF-α in a dose-dependent manner (Fig. 2). Taken together, these results show that, despite some functional divergence among the p160 members [23], all tested coactivators were able to enhance κB-mediated transcription upon a physiological stimulus in cell culture, indicating that coactivators may play an important role in controlling NF-κB target gene expression.

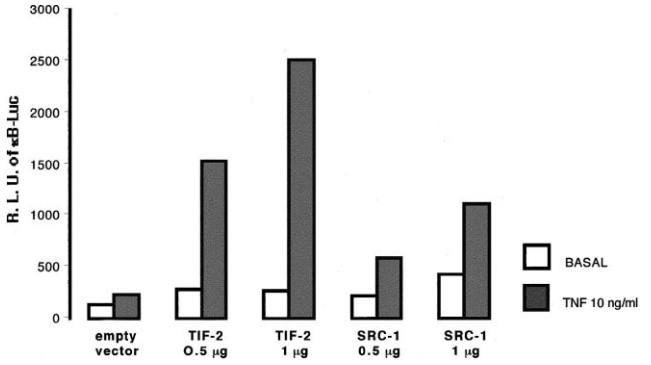

Fig. 2. Role of coactivators on κB-mediated transcription. Cells were transfected with 500 ng of κB-Luc reporter plasmid and 500 ng of RSV-β-GAL along with the indicated amounts of coactivators pCR3.1-SRC-1 or pSG5-TIF-2. The total amount of DNA was 3 μg. Diagram bars represent the average of triplicate values. Similar results were obtained in three independent experiments. S.D. was less than 10%.

3.2. *RAC3 coactivator interacts with the Rel-A heterocomplex and requires activated NF-κB for κB transactivation*

In order to elaborate on the preceding findings, we wanted to know if RAC3 is capable of physically interacting with the active NF-κB complex. We therefore performed co-immunoprecipitation experiments with HeLa cells. In these experiments, the cells were stimulated with TNF-α in order to ensure high levels of active NF-κB complex, for putative interaction with the coactivator. As shown in Fig. 3A, Rel-A protein was readily detected in the RAC3 immunoprecipitate. These data suggest that RAC3 and Rel-A may be part of the same protein complex by direct binding or through an indirect association mediated by other proteins.

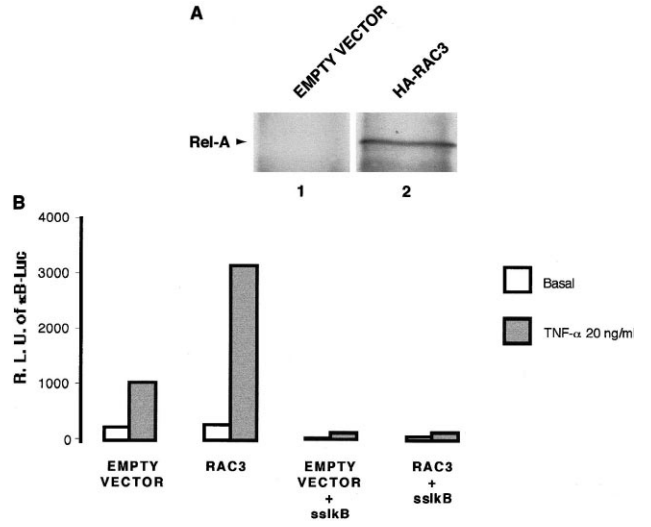

Fig. 3. A: Western analysis for Rel-A in RAC3 immunoprecipitates. Anti-HA immunoprecipitates from cells transfected with either empty vector (lane 1) or pCMX-HA-tagged RAC3 (lane 2) and stimulated with 10 ng/ml of TNF-α for 45 min are shown. B: Effect of RAC3 on κB-mediated transcription in the absence of active NF-κB. Cells were transfected with 500 ng of κB-Luc reporter plasmid, 500 ng of RSV-β-GAL, 0.5 μg of pCMX-RAC3 or empty vector, and 1 μg of ssIκB expression vector and stimulated as indicated. Diagram bars represent the RLU of assay. Similar results were obtained in three independent experiments. A similar effect of ssIκB was also observed in cells transfected with 1 μg of pCMX-RAC-3 and 500 ng of ssIκB.

In order to determine if the effect of RAC3 on NF-κB-mediated transcription is specifically mediated by the activated form of NF-κB, we transiently transfected HeLa cells with κB-Luc reporter along with vectors expressing RAC-3 and the mutated inhibitor of NF-κB (ssIκB). This mutant is not susceptible to phosphorylation and hence, does not undergo proteolysis upon TNF-α stimulation and, for that reason, constitutively suppresses NF-κB activation. As shown in Fig. 3B, overexpression of ssIκB entirely inhibited TNF-α-induced NF-κB transactivation. In addition, abundant RAC3 was un-

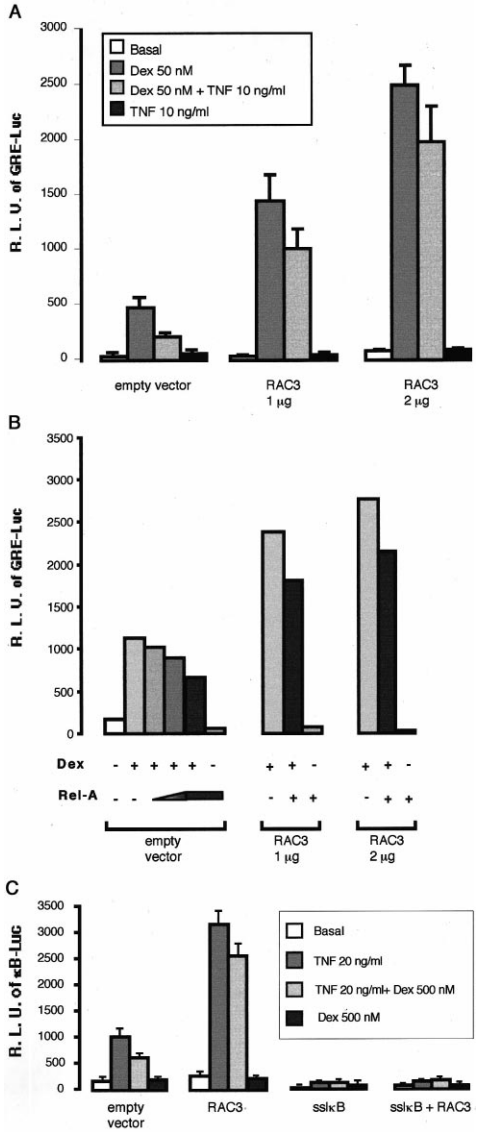

Fig. 4. Role of RAC3 in the mutual GR/NF-κB transrepression. A: Cells were transfected with 500 ng of the GRE reporter MMTV-Luc, 500 ng of RSV-β-GAL and 1 μg of pCMX-RAC3 or the empty vector and stimulated with 50 nM of DEX and 10 ng/ml of TNF-α, as indicated. Diagram bars are the average ± S.D. of three independent experiments. B: Cells were transfected with 500 ng of the GRE reporter, 500 ng of RSV-β-GAL, increasing doses of Rel-A expression vector (200 ng, 500 ng, 1 μg) and RAC3 expression vector and stimulated with 500 nM of DEX as indicated. Diagram bars represent the RLU of assay. C: Cells were transfected with 500 ng of κB-Luc reporter, 500 ng of RSV-β-GAL, 1 μg of pCMX-RAC3 or the empty vector, 1 μg of ssIκB expression vector and stimulated with TNF-α and DEX, as indicated. Diagram bars represent the average ± S.D. of three independent experiments.

able to overcome this suppression. These results, together with the ability of the coactivator to enhance κB promoter activity in stimulated cells, demonstrate that the effect of RAC3 on κB-dependent transcriptional activity is absolutely dependent on the presence of an active form of NF-κB.

3.3. *RAC3 coactivator rescues both GRE- and κB-dependent transcription from the mutual inhibitory effect between TNF-α-induced NF-κB and GR*

Although very low doses of TNF-α, such as 0.02 ng/ml, may enhance the GR transactivation [33], this cytokine and glucocorticoid antagonize each others effect in the NF-κB/GR-pathways, [17–19]. A physical interaction between GR and NF-κB may partially account for the mutual transrepression, however, the induction of IκB expression by GR is not sufficient to explain Rel-A-mediated downregulation of GR transactivation.

In order to determine if competition for limiting quantities of endogenous RAC3 might be a possible mechanism underlying the mutual GR/NF-κB antagonism, we analyzed GRE as well as κB-mediated transcription under RAC3-saturated conditions. These experiments were performed with or without the addition of DEX and/or TNF-α.

First, we examined the role of RAC-3 on NF-κB transrepression of GRE-dependent transcription. We co-transfected HeLa cells with GRE reporter plasmid along with 'empty' control plasmid or RAC3 expression vector and analyzed GR transcriptional activity of cells treated with DEX, TNF-α or a combination of both.

As shown in Fig. 4A, co-treatment with TNF-α (10 ng/ml) inhibited GRE-mediated transactivation stimulated with 50 nM DEX. The presence of abundant RAC3 reversed squelching for the coactivator and resulted in a significantly reduced TNF-α-induced transrepression (Fig. 4A). Similar results were obtained with non-stimulated cells that were transfected with an expression vector for Rel-A protein in a dose-dependent manner (Fig. 4B). Increasing amounts of RAC3 reversed the effect for the maximal dose of Rel-A applied in the experiment.

We wanted to determine if RAC3 is also capable of overcoming the GR inhibition of TNF-α-stimulated NF-κB transcription and for that purpose we employed a similar approach. Fig. 4C shows that overexpression of RAC3 again, was sufficient to overcome NF-κB/GR transrepression. However, in this experiment, much higher levels of glucocorticoids (500 nM of DEX) were required to lower κB-mediated transactivation ($42 \pm 10\%$) while in the previous experiment, the addition of DEX at 50 nM was sufficient to significantly induce GRE-transcriptional activation (Fig. 4A).

Treatment with DEX alone had no effect on κB-transcriptional activity, which was not affected by the presence of abundant RAC3 (Fig. 4C). In addition, co-transfection with ssIκB expression vector reduced NF-κB transcriptional activity almost to basal levels even under conditions of Rac-3 overexpression. Taken together, these results indicate that the restoration of the DEX effect by RAC3 requires the active form of NF-κB and is not due to the binding of RAC3 to another transcription factor.

Since overexpression of RAC3 does not overcome the inhibitory effect of ssIκB, and RAC3 specifically enhances the transcriptional activity of activated, inhibitor-depleted NF-κB; we believe in the existence of an additional mechanism responsible for the mutual antagonism of glucocorticoids and cytokines on κB/GRE-transcription. Such a mechanism is sensitive to the cellular levels of RAC3 and is not constrained by the activation of IκB proteolysis. Under physiological conditions where both GR and NF-κB pathways are in need of coactivators, the model supports the action of RAC3 as a limiting cofactor. Thus, the competitive squelching of RAC3 by both activated pathways may contribute to the mutual transrepression.

According to other publications, there is strong evidence that the induction of IκB by glucocorticoids is not a general molecular mechanism that may explain the NF-κB transrepression [28,29]. Moreover, it was previously shown that in U937 cells, glucocorticoids do not prevent neither the nuclear localization of NF-κB nor its binding to DNA, but rather change the complex into a transcriptionally inert form [34]. While some authors have suggested that coactivators may constitute a limiting factor for transcriptional activity, others have shown that one coactivator may stabilize the transcriptionally inactive complex GR/NF-κB [28].

In view of these evidences, it becomes clear that the mutual transrepression between GR and NF-κB involves more than one mechanism which probably depends of the cellular scenario, promoter type, coactivator levels and physiological context.

Our results support the idea that the competition for coactivators is one of the molecular mechanisms that explain GR/NF-κB mutual transrepression, where RAC3, being a limiting factor, plays a critical role as an NF-κB and GR coactivator.

Acknowledgements: We thank Dr. G. Cadwell, Dr. M. Karin, Dr. J. DiDonato, Dr. H. Gronemeyer, Dr. P. Chambon and Dr. R. Evans for providing plasmid constructs. We are especially grateful to Dr. A. Kornblihtt and Dr. O.A. Coso for comments about the manuscript. This work has been supported by grants from the University of Buenos Aires (UBA), the Argentine National Research Council (CONICET) and Fundación Antorchas, Argentina.

References

[1] Ghosh, S., May, M.J. and Koop, E.B. (1998) Annu. Rev. Immunol. 16, 225–260.
[2] Baeuerle, P.A. and Baltimore, D. (1996) Cell 87, 13–20.
[3] Baldwin, A.S. (1996) Annu. Rev. Immunol. 14, 649–681.
[4] Kaltschmidt, B., Kalschmidt, C., Hehner, S.P., Drogue, W. and Schmitz, M.L. (1999) Oncogene 18, 3213–3225.
[5] Mukhopadhyay, T., Roth, J.A. and Maxwell, S.A. (1996) Int. J. Oncol. 9, 715–720.
[6] Liu, Z., Hsu, H., Goeddel, D.V. and Karin, M. (1996) Cell 87, 565–576.
[7] Beg, A. and Baltimore, D. (1996) Science 274, 782–784.
[8] Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. and Verma, I.M. (1996) Science 274, 787–789.
[9] Chu, Z.-L., McKinsey, T., Liu, L., Gentry, J.J., Malim, M.H. and Ballard, D.W. (1997) Proc. Natl. Acad. Sci. USA 94, 10057–10062.
[10] Wang, C., Mayo, M.W., Korneluk, R.G., Goeddel, D.V. and Baldwing Jr., A.S. (1998) Science 281, 1680–1683.
[11] Besedovsky, H., Del Rey, A., Sorkin, E. and Dinarello, C.A. (1986) Science 233, 652–654.
[12] Sapolsky, R., Rivier, C., Yamamoto, G., Plotsky, P. and Vale, W. (1987) Science 238, 22–24.
[13] Snyder, D.S. and Unanue, E.R. (1982) J. Immunol. 129, 1803–1805.
[14] Gillis, S., Crabtree, G.R. and Smith, K. (1979) J. Immunol. 123, 1624–1631.
[15] Cupps, T.R. and Fauci, A.S. (1982) Immunol. Rev. 65, 133–155.
[16] Hollenberg, S.M. and Evans, R.M. (1988) Cell 55, 899–906.

[17] Scheiman, R.I., Gualberto, A., Jewell, C.M., Cdlowsky, J.A. and Baldwin, J.R. (1995) Mol. Cell. Biol. 15, 943–953.
[18] Scheinman, R.I., Cogswell, P.C., Lofquist, A.K. and Baldwin Jr., A.S. (1995) Science 270, 283–286.
[19] Auphan, N., DiDonato, J.A., Rosette, C., Helmberg, A. and Karin, M. (1995) Science 270, 286–290.
[20] Torchia, J., Glass, C.K. and Rosenfeld, M.G. (1998) Curr. Opin. Cell Biol. 10, 373–383.
[21] Xu, L., Glass, C.K. and Rosenfeld, M.G. (1999) Curr. Opin. Genet. Devel. 9, 140–147.
[22] DiCroce, L., Okret, S., Kersten, S., Gustafsson, M.P., Wahli, W. and Beato, M. (1999) EMBO J. 18, 6201–6210.
[23] McKenna, N., Lanz, R.B. and O'Malley, B.W. (1999) Endocrine Rev. 20, 321–344.
[24] Na, S.-Y., Lee, S.-K., Han, S.-J., Choi, H.-S., Im, S.-Y. and Lee, J.W. (1998) J. Biol. Chem. 273, 10831–10834.
[25] Zhong, H., Voll, R.E. and Ghosh, S. (1998) Mol. Cell 1, 661–671.
[26] Sheppard, K.-A., Rose, D.W., Haque, Z.K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M.G., Glass, C.K. and Collins, T. (1999) Mol. Cell. Biol. 19, 6367–6378.
[27] Sheppard, K.-A., Phelps, K.M., Williams, A.J., Thanos, D., Glass, C.K., Rosenfeld, M.G., Gerritsen, M.E. and Collins, T. (1998) J. Biol. Chem. 273, 29291–29294.
[28] McKay, L.I. and Cidlowski, J. (2000) Mol. Endocrinol. 14, 1222–1234.
[29] De Bosscher, K., Berghe, W.V., Vermeulen, L., Plaisance, S., Boone, E. and Haegeman, G. (2000) Proc. Natl. Acad. Sci. USA 97, 3919–3924.
[30] Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauler, G., Kallioniemi, O.P., Trent, J.M. and Meltzer, P.S. (1997) Science 277, 965–968.
[31] Lanz, R.B., McKenna, N.J., Onate, S.A., Albrecht, U., Wong, J., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1999) Cell 97, 17–27.
[32] Voegel, J.J., Heine, M.J.S., Tini, M., Vivat, V., Chambon, P. and Gronemeyer, H. (1998) EMBO J. 17, 507–519.
[33] Costas, M., Trapp, T., Páez Pereda, M., Sauer, J., Rupprecht, R., Nahmod, V., Reul, J.M.H.M., Holsboer, F. and Arzt, E. (1996) J. Clin. Invest. 98, 1409–1416.
[34] Liden, J., Rafter, I., Truss, M., Gustafsson, J.-A. and Okret, S. (2000) Biochem. Biophys. Res. Commun. 273, 1008–1014.