Instituto Juan March
de Estudios e Investigaciones

57 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

NF-κB/IκB Proteins. Their Role in Cell Growth, Differentiation and Development

Organized by

R. Bravo and P. S. Lazo

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D. Baltimore
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INTRODUCTION

Rodrigo Bravo and Pedro S. Lazo

Ten years ago the first paper reporting the existence of a kappa immunoglobulin enhancer binding protein was published and called NF-κB. This binding function presented a stage specific pattern of activation in B cells which led to a model that included an inhibitory protein (IκB) which could retain NF-κB in the cytoplasm. Upon activation of the cell, the inhibitor would be inactivated and NF-κB translocated to the nucleus to activate gene transcription.

Since then, NF-κB has been found to be an ubiquitous transcription factor composed of dimers and several homologous proteins have been identified, e.g., p50, p52, c-Rel, RelB, RelA (p65). The term Rel/NF-κB now refers to a family of closely related dimeric complexes which are able to regulate specific gene transcription. In the last six years, emphasis has been placed on isolating and cloning the various members of the Rel/NF-κB family, characterizing their structure and their interaction with DNA.

The family of inhibitory proteins, which includes the proto-oncogene Bcl-3, has also been the subject of active research as well as the study of the mechanisms by which different activators trigger NF-κB activation. It is now clear that phosphorylation and ubiquitin mediated proteolysis are implicated in the mechanism of activation of NF-κB. However, it remains unclear which kinases and proteases are responsible for these processes.

The important role of NF-κB/IκB proteins in growth regulation and differentiation can be deduced from their involvement in the transcriptional activation by growth factors, the oncogenic activities of some of the family members, and their activation by mitogens. However, the role of each individual protein has not been clearly established probably because of the ability of NF-κB proteins to substitute for each other in the heterodimeric complexes.

The workshop dealt with the most recent advances in this active field of research, from the molecular level to the most complex biological models. Thus, it was discussed the characterization of the molecular interaction between DNA and NF-κB proteins using crystallography data as well as biochemical studies on the interaction of the different NF-κB proteins and their inhibitors, including the new inhibitor IκBε.

From the data presented at the workshop, it is apparent that signal transduction from the cell membrane to NF-κB is rather complex, with different pathways being implicated.

It also appears that different kinases may be involved in the phosphorylation of IκB proteins and that these inhibitory proteins play different roles in the activation process of NF-κB.

An important part of the workshop was devoted to the description of the characterization of transgenic and KO mice. This has been one of the major advances reported in the workshop. So far, all the mutant mice have survived,confirming the superimposing functions of the family members.

Future studies of double or even triple KO mice will probably yield more clear cut answers to the questions concerning the functions of these molecules. As it was observed soon after the discovery of NF-kB proteins, homologs are also present in insect cells where they play an important role in development. A session of the workshop focused on these proteins. The similarities between the Rel/NF-κB proteins from higher organisms and those from insects have become more apparent, including a possible role in defence mechanisms of insects for new NF-κB proteins.

We would like to thank the speakers for their very open and candid presentations and informal discussions; they were all very engaging. We are also very grateful to the Fundación Juan March for the excellent support and organization of this meeting.

Rodrigo Bravo
Pedro S. Lazo

Session 1:

Chairperson: Inder M. Verma

The Structure of the p50 NFκB homodimer bound to a κB element.

Paul B. Sigler.- Department of Molecular Biophysics & Biochemistry and the Howard Hughes Medical Institute, Yale University, New Haven, CT. (USA).

The crystal structure of the Rel homology region (RHR) of the NF-κB p50 homodimer bound to a symmetrical idealized ten-base pair κB element has been refined to 2.3Å The RHR is composed of two antiparallel beta sheet domains. Each domain provides 2 loops to the DNA-binding surface. A fifth loop is provided by the linker between the domains. Together, the dimer presents ten loops to the major groove of the κB element. There are no β-sheet or α-helical components in the recognition interface. The DNA is essentially unbent. There are no interactions with the minor groove edges of the bases, however, the two antiparallel α-helices (possibly unique to p50) that occur in a nonconserved seventy-aminoacid segment in the first domain flank the minor groove at the center of the element and interact with DNA backbone. The DNA is not significantly bent. The smaller C-terminal domains forms a symmetrical beta-sandwich dimer interface.

The overall structure of the p50 homodimer superficially resembles an immunoglobulin, however, the DNA interactive surfaces are in the center of the dimer rather than at the hapten-binding tips of the immunoglobulin analogue. Careful analysis of the folding pattern supports a greater similarity with primitive antimicrobials rather than with an immunoglobulin fold.

The signature sequence of the κB element, GG.....CC is explained by the intraction of a conserved pair of well buttressed arginine residue with the conserved flanking guanine bases. Contact with the variable internal bases is made by polar side chains some of whose hydrophobic stems interact with the hydrophobic residues of the dimer interface. Thus, changing dimer/ partners (eg., p50/p50 to p50/p65) could change the trajectory of these side chains and hence the position of their polar tips. This might explain the need for specific internal base sequences to support binding and orientation of specific dimer pairs.

The two domains are linked by a ten residue segment which is involved in DNA binding but would otherwise be flexible. It is not unlikely that in the absence of DNA the domains could reorient themselves to interact with a regulatory protein such as IκB.

Studies of NF-κB functions and activation. David Baltimore, Amer Beg, Bruce Horwitz and Ilana Stančovski, MIT

We have been analyzing NF-κB's functions by studying the consequences of knocking out the mouse genes for the p50, p65 and I-κBα polypeptides. We have recently been concentrating on the role of p65 which has to be analyzed in embryonic fibroblasts or by adoptive transfer protocols because of the embryonic lethality. We have shown that in the p65-/- cells, but not in normal or heterozygous cells TNF treatment leads to lethality. Expression of p65 can overcome the lethality. Thus NF-κB appears to have as one of its functions countering apoptotic signals. We have also found that B and T cells lacking p65 have fairly normal properties, responding effectively to mitogenic signals in vitro. Thus, p50 and c-Rel have keys roles in the activation of B cells but p65 may not have an important role at that stage of development. We have been seeking kinases that phosphorylate IκBα and have found in HeLa cells an activity that is about 300 kD and which quite specifically phosphorylates the serines in the N-terminus of IκB. Purification of that activity is in progress.

Characterization of NF-κB knockout mice

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Mice with a targeted disruption of RelB, a member of the Rel/NF-kB family of transcription factors, have multifocal, mixed inflammatory cell infiltration in several organs, myeloid hyperplasia, and splenomegaly due to extramedullary hematopoiesis. To elucidate the cellular requirements for this complex phenotype, we have bred RelB-deficient (RelBKO) animals to two strains of immunodeficient mice, RAG-1-deficient (RAG-1KO, lacking B and T cells) and Nur77/N10-transgenic mice (Nur77/N10TG, lacking only T cells). We also generated mutant mice deficient in both RelB and the p50 subunit of NF-kB (p50KO, multiple defects in B cell function). RelBKORAG-1KO and RelBKONur77/N10TG mice are disease free, while RelBKOp50KO double-mutant animals develop an even more severe phenotype despite the absence of B cells in the inflammatory infiltrates. Thus, both multiorgan inflammation and myeloid hyperplasia in RelB-deficient mice are T cell-dependent whereas B cells are not crucially involved.

We have shown that RelB-deficient mice are highly susceptible to infection by Listeria monocytogenes. They are also unable to clear lymphocytic choriomeningitis virus, indicating a defective cytotoxic T cell response. RelB-deficient animals have increased serum IgM and IgE and impaired production of antigen-specific IgG in the absence of T cell help. Negative and positive selection is not impaired in these animals.

The *nfkb2* gene produces the p52 protein after proteolytic cleavage of the p100 precursor. The p52 product might act as an alternative subunit of NF-kB, whereas the p100 precursor is thought to be a functional IκB member. To assess the function of p100, we generated mice lacking the precursor, but still containing the p52 product by gene targeting. Mice with homozygous deletion in the C-terminal ankyrin repeats of NF-kB2 showed extensive gastric hyperplasia with lymphocytic infiltration, resulting in early postnatal death. Mice deficient in the precursor also displayed enlarged lymph nodes and increased lymphocyte proliferation *in vitro* in response to several external stimuli. Dramatic induction of the nuclear κB-binding activity composed of p52-containing complexes was found in every tissue examined of the p100-deficient mice.

Probing for Physiologic Roles of NF-κB/IκB Proteins with Gene Knock-Out Mice.

Ulrich Siebenlist

NF-κB activity is central to immune- and stress- responses and this activity is controlled by a family of ankyrin-containing IκB proteins. The primary NF-κB complex in most cells is a p50/p65 heterodimer, which is rapidly released from the cytoplasmic IκB–α inhibitor upon cellular stimulation with many agents. Once released, NF-κB is free to enter nuclei and initiate transcriptional activation of target genes. It often does so by cooperative interaction with other factors, including an unusual interaction we detected with the Serum Response Factor. The mechanism by which NF-κB is released from IκB-α in the cytoplasm involves signal-induced phosphorylation at Serines 32 and 36 in the NH2-terminal part of IκB-α. This phosphorylation is mediated by a kinase-containing complex whose activation is somehow dependent on ubiquitination. Pathways leading to activation of this kinase complex are yet to be worked out, but may involve several distinct routes, including a Raf kinase-dependent pathway as well as other recently recognized MAPKinase pathways. Induced phosphorylation of both Serines marks IκB-α for ubiquitin-ligation, primarily on Lysines 21 and 22. Finally, multiubiquitinated IκB-α is degraded by proteasomes, a step which also appears to require the COOH-terminal part of IκB-α, a part of the protein which contains PEST sequences and constitutive phosphorylation sites. Our results indicate that the NH2- and COOH-terminal parts of IκB-α are both necessary and sufficient to mediate the signal-induced rapid proteolysis.

NF-κB activity is controlled not only by IκB-α and the functionally related IκB-β, but also by the other members of this family, including the oncoprotein Bcl-3. Prior research has established that Bcl-3, unlike IκB-α and -β, can easily localize to the nucleus, where it appears to act as a positive regulator of transactivation, rather than a negative one. Several mechanisms have been proposed for this effect, primarily involving interaction with the p50 and p52 homodimeric complexes. However, nothing is known about the physiologic context in which p50 and/or p52 homodimers may be relevant or in which the regulatory potential of Bcl-3 may be utilized, nor have target genes been identified . To address these questions we have generated gene 'knock-out' mice. These mice exhibit various specific immune defects.

1. Brown, K. et al. (1995) Science 267:1485.
2. Baldi, L. et al. (1996) J. Biol. Chem., 271:376.
3. Franzoso, G. et al. (1996). EMBO, in press.

Rel deficient mice exhibit multiple immune system defects.

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Rel, the product of the *c-rel* protooncogene, expression of which is largely restricted to hemopoietic cells, is one of the subunits that comprise the NF-κB-like family of transcription factors. To better understand the physiological role of Rel, gene targeting was used to generate mice with an inactivated *c-rel* gene (1). The development of all hemopoietic lineages in these mice are normal, but mature hemopoeitic cells exhibit multiple defects during cellular activation. While single mitogenic agents fail to induce $Rel^{-/-}$ B cell proliferation, certain combinations of stimuli and growth factors can in part overcome this proliferative block. The inability of $Rel^{-/-}$ T cells to proliferate in response to antigen and co-receptor engagment can be rescued by the addition of exogeneous IL-2. Analysis of gene expression reveals that $Rel^{-/-}$ deficient T cells and macrophages are defective in the expression of multiple cytokines. Stimulated $Rel^{-/-}$ T cells produce reduced levels of IL-2, IL-3 and GM-CSF. $Rel^{-/-}$ macrophages display a more complex mutant phenotype, with TNF-α production reduced, but GM-CSF, G-CSF and IL-6 expression elevated above that of normal cells. Consistent with the defects in the activation response of mature hemopoietic cells, $Rel^{-/-}$ mice fail to mount effective humoral and cell mediated immune responses to a number of pathogens. Collectively, these findings indicate that Rel functions both as a positive and negative transcriptional regulator that is important for cytokine expression and lymphocyte proliferation.

1. Kontgen F., Grumont, R.J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D and Gerondakis,S.(1995). Genes and Develop. 9:1965-1977.

The Role of the NF-κB2 Gene and its Encoded Protein (p52) *In Vivo*

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The NF-κB family of transcription factors is implicated in the expression of genes responsible of lymphocyte activation, acute phase response and inflammation in the immune system. One of its members, the NF-κB2 gene has been identified some years ago as a candidate proto-oncogene since its involvement in some cases of human B-cell lymphoma. This gene encodes for a precursor protein of 100 KD (p100) that remains in the cytoplasm and upon proteolytic processing gives origin to p52, the subunit that will translocate to the nucleus and bind DNA.
More recently, several groups have shown that NF-κB2 is also implicated in the development of multiple myelomas, T-cell lymphomas and in particular cutaneous T-cell lymphomas. The rearrangements found in this locus showed overexpression and deletions of the carboxi terminal domain of the precursor protein.
To further elucidate the function of NF-κB2 we have generated mutant mice for this gene by homologous recombination. For that purpose a genomic DNA library of Sv/129 mouse strain was screened using a mouse NF-κB2 cDNA probe.
Several clones were isolated spanning a region of 18 Kb containing the promoter and the first 20 exons of this gene. As a targeting strategy two fragments of 10 and 4 Kb respectively were subcloned in the targeting vector and the neomycin phosphotransferase gene under the control of the mouse PGK promoter was inserted in exon 4 disrupting the open reading frame of the NF-κB2 gene. This construct was electroporated in embryonic stem (ES) cells and several clones that underwent homologous recombination at the NF-κB2 locus were obtained. Two NF-κB2 (+/-) clones were used to generate heterozygous and subsequently homozygous (-/-) mice.
A detailed analysis of the phenotype of the NF-κB2 null mice will be presented.

Session 2:

Chairperson: Nancy Rice

Malignant Transformation of Chicken Spleen Cells by the v-Rel Oncoprotein. Thomas D Gilmore, Biology Department, Boston University

One of the primary focuses of my laboratory is understanding the mechanism by which the v-Rel oncoprotein encoded by the highly oncogenic avian Rev-T retrovirus malignantly transforms and immortalizes chicken lymphoid cells in vitro and in vivo (reviewed in refs 3 and 4).

v-Rel is highly mutated as compared to c-Rel, but it appears that the primary change that is important for the oncogenicity of v-Rel is the deletion of C-terminal amino acids in v-Rel as compared to c-Rel. In addition to conferring increased oncogenicity to v-Rel, this deletion removes a strong C-terminal c-Rel activation domain and allows v-Rel to enter the nucleus of many cells (2,5,7). Thus, the overall structure of v-Rel is such that there is an N-terminal Rel homology domain that has sequences involved in DNA binding, the formation of heterodimers and homodimers, and nuclear localization, and a truncated C-terminal domain that has transcription activation sequences. Of note, the C-terminal deletion in v-Rel is similar to the changes that have occurred in certain *rel* family genes due to chromosomal rearrangements in human lymphoid cancers.

In transformed cells, v-Rel interacts with a number of cellular proteins, including NF-κB p100, NF-κB p105, c-Rel and IκB-α (1,2,9). In addition, homodimers of v-Rel that can bind to DNA are also present in cells. Using mutants of v-Rel, we have determined functions that are important for transformation by v-Rel. Numerous studies have shown that DNA binding by v-Rel is essential for its transforming function; for example, temperature-sensitive (ts) transforming mutants of v-Rel are ts for DNA binding (10). Furthermore, it is likely that homodimers of v-Rel are the important effector molecules for transformation. For example, one v-Rel mutant, v-SPW, is non-transforming and is defective for the formation of homodimers, but v-SPW can still form functional heterodimers with NF-κB p105/p50 and p100/p52 (12). In addition, certain ts v-Rel mutants cannot form homodimers and bind DNA at the non-permissive temperature, but can still form heterodimers at the non-permissive temperature (1,6,12). The interaction of v-Rel with IκB-α does not appear to be relevant to transformation, since a v-Rel mutant with a deletion of sequences in the primary nuclear localization signal does not interact with IκB-α, but transforms chicken spleen cells as efficiently as wild-type v-Rel (12).

Sequences C-terminal to the Rel homology domain also are important for transformation by v-Rel. The study of several deletion mutants indicates that C-terminal sequences that contain weak transcription activation domains are necessary for transformation by v-Rel (8). To determine how these sequences may activate transcription, or possibly to identify other functions residing in the C-terminal half of v-Rel, we have performed a two-hybrid screen with C-terminal sequences of v Rel. We have identified two cDNAs from a human B-cell library that encode novel proteins that specifically interact with C-terminal sequences of v-Rel. We are currently characterizing these v-Rel-interacting proteins to determine their effect on v-Rel and their possible relevance to transformation by v-Rel.

v-Rel and derivatives of chicken c-Rel are the only Rel/NF-κB proteins that are consistently transforming in vitro. Although rearrangements in c-*rel* and in the gene encoding NF-κB p100 have been detected in a number of human lymphoid cancers, these proteins have not been demonstrated to be transforming in vitro. We have recently found that heterodimers between v-Rel mutant v-SPW and chicken NF-κB p52 can induce the malignant transformation of chicken spleen cells (12). This result demonstrates for the first time that heterodimeric Rel complexes can induce transformation. In addition, we have found that truncated p100 proteins from human lymphoid tumors activate transcription in yeast, whereas normal p52 and p100 proteins do not.

We have also been using the ts v-Rel mutants to analyze immortalization of chicken spleen cells by v-Rel. When ts v-Rel-transformed cells are shifted to the

nonpermissive temperature, these cells undergo apoptosis and IκB-α undergoes proteolysis (13). We have recently found that apoptosis in these cells can be blocked by overexpression of Bcl-2, suggesting that v-Rel immortalizes cells through a Bcl-2-dependent pathway (11). In contrast, the ICE protease inhibitor CrmA does not block apoptosis when ts v-Rel-transformed cells are shifted to the nonpermissive temperature; however, CrmA does block an N-terminal processing of IκB-α that occurs in these cells. Our preliminary results indicate that IκB-α is a substrate for an ICE-related protease.

References

1. Capobianco AJ, D Chang, G Mosialos & TD Gilmore. 1992. p105, the NF-κB p50 precursor, is one of the cellular proteins complexed with the v-Rel oncoprotein in transformed chicken cells. J Virol 66 3758-3767

2. Capobianco A, DL Simmons & TD Gilmore. 1990. Cloning and expression of a chicken c-rel cDNA: unlike $p59^{v\text{-}rel}$, $p68^{c\text{-}rel}$ is a cytoplasmic protein in chicken embryo fibroblasts. Oncogene 5 257-266

3. Gilmore TD 1991. Malignant transformation by mutant Rel proteins. Trends Genet 7 318-322

4. Gilmore TD, DW White, S Sarkar & S Sif. 1995. Malignant transformation of cells by the v-Rel oncoprotein. In, The DNA Provirus: Howard Temin's Scientific Legacy (eds GM Cooper, R Greenberg Temin, and B Sugden), American Society for Microbiology, Washington D.C., pp 109-128

5. Kamens J, P Richardson, G Mosialos, R Brent & TD Gilmore. 1990. Oncogenic transformation by vRel requires an amino-terminal activation domain. Mol Cell Biol 10 2840-2847

6. Mosialos G & TD Gilmore. 1993. v-Rel and c-Rel are differentially affected by mutations at a consensus protein kinase recognition sequence. Oncogene 8 721-730

7. Richardson P & TD Gilmore. 1991. vRel is an inactive member of the Rel family of transcriptional activating proteins. J Virol 65 3122-3130

8. Sarkar S & TD Gilmore. 1993. Transformation by the vRel oncoprotein requires sequences carboxy-terminal to the Rel homology domain. Oncogene 8 2245-2252

9. Sif S & TD Gilmore. 1993. NF-κB p100 is one of the high-molecular-weight proteins associated with the v-Rel oncoprotein in transformed chicken spleen cells. J Virol 67 7612-7617

10. White DW & TD Gilmore. 1993. Temperature-sensitive transforming mutants of the v-rel oncogene. J Virol 67 6876-6881

11. White DW & TD Gilmore. 1996. Bcl-2 and CrmA have different effects on transformation, apoptosis, and the stability of IκB-α in chicken spleen cells transformed by temperature-sensitive v-Rel oncoproteins. Oncogene 12 in press

12. White DW, GA Pitoc & TD Gilmore. 1996. Interaction of the v-Rel oncoprotein with NF-κB and IκB proteins: heterodimers of a transformation-defective v-Rel mutant and NF-κB p52 are functional in vitro and in vivo. Mol Cell Biol 16 1169-1178.

13. White DW, A Roy & TD Gilmore. 1995. The v-Rel oncoprotein blocks apoptosis and proteolysis of IκB-α in transformed chicken spleen cells. Oncogene 10 857-868

Tetracycline-regulated expression of v-Rel and c-Rel: Effects on cell proliferation and gene expression

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Our laboratory is interested in the transcriptional activity of the v-Rel and c-Rel proteins and their role in cell proliferation and transformation. Previous studies from our group and others identified a transcription activation domain in v-Rel (1, 2) that overlaps with sequences required for cell transformation (3). We also reported the functional interaction of this activation domain and that of c-Rel with general transcription factors (4). Our recent mapping experiments further support an important role for the transcriptional activity of v-Rel in cell transformation (5).

In this study, we developed tetracycline-regulated cell systems to characterize the effects of v-Rel and c-Rel on cell proliferation and gene expression. We report that high level expression of c-Rel decreased the progression of cells from G1 into S phase, and altered cell morphology in a way that correlated with its subcellular localization. Prolonged expression of the protein promoted the accumulation of cells in a sub-G0/G1 population undergoing death by apoptosis. The phenotype of c-Rel in our cell system agrees with analyses by Abbadie et al. in developing chicken embryos, showing a correlation between c-*rel* expression and apoptosis in several non-hematopoietic tissues (6). In sharp contrast, v-Rel increased G1/S phase transition in our cell system whereas its down-regulation in transformed lymphoid cells resulted in programmed cell death. This observation is consistent with the studies of White et al. (7). The fact that the phenotype of c-Rel differed markedly from that observed with v-Rel points to the possible activation of specific cellular genes.

Investigation into the mechanisms involved in c-Rel-mediated growth arrest revealed a marked decrease in E2F DNA-binding activity and the accumulation of Rb in its hypophosphorylated form. *In vitro* kinase assays demonstrated the inhibition of Cdk2 kinase activity in response to c-Rel expression, while immunoblots indicated the accumulation of the p21/Waf1 Cdk-inhibitor. Northern blot analysis during a time course of c-Rel induction showed the accumulation of p21 transcripts with kinetics that paralleled those for c-*rel* and *mad-3/ikba*. The contribution of p53-dependent and p53-independent pathways to this process is under investigation.

mRNA differential display analysis of cellular gene expression in our cell systems also revealed the induction and repression of specific cellular genes in response to v-Rel and c-Rel. Here we report the isolation of a cDNA clone for a c-Rel-responsive gene from a human spleen library. Northern blot analysis showed hybridization of this clone to a 5.8 kb transcript that was induced with kinetics that paralleled those for c-*rel* and *mad-3/ikba*. Expression of this transcript was also observed in lymphoid cell lines and tissues. Its characterization as a c-Rel-regulated gene is in progress. Ongoing and future studies will help to further clarify the pathways through which Rel proteins affect cell proliferation and the role of their transcriptional activity in this process.

(1) Ishikawa, H., M. Asano, T. Kanda, S. Kumar, C. Gélinas, and Y. Ito (1993) Oncogene 8: 2889-2896.
(2) Walker, W.H., B Stein, P.A. Ganchi, J.A. Hoffman , P.A. Kaufman, D.W. Ballard, M. Hannink, and W.C. Greene (1992) J. Virology. 66: 5018-5029.
(3) Sarkar, S., and T.D. Gilmore (1993) Oncogene 8: 2245-2252.
(4) Xu, X., C. Prorock, H. Ishikawa, E. Maldonado, Y. Ito, and C. Gélinas (1993) Mol. Cell. Biol. 13: 6733-6741.
(5) Agnès, F., Y. Hu, X. Xu and C. Gélinas. In preparation.
(6) Abbadie, C., N. Kabrun, F. Bouali, J. Smardova, D. Stéhelin, B. Vandenbunder, and P.J. Enrietto (1993) Cell 75: 899-912.
(7) White, D.W., A. Roy and T.D. Gilmore (1995) Oncogene 10: 857-868.

The v-*rel* oncogene promotes malignant T cell leukemia/lymphoma in transgenic mice

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The oncogene product from the avian reticuloendotheliosis virus strain T, v-Rel, is a member of the Rel/NF-kB family of transcription factors. The mechanism by which v-Rel induces oncogenic transformation remains unclear. Several attempts to transform mammalian cells with v-Rel have failed, suggesting that v-Rel transformation may be a species-specific event. However, we have demonstrated that v-Rel but not a truncated c-Rel, expressed under the control of the *lck* promoter, efficiently induced malignancies in transgenic mice. Most of the animals died before 10 months of age and developed immature, multicentric aggressive T cell leukemia/lymphomas. Most tumors contain $CD4^+CD8^+$ cells or $CD4^-CD8^+$ cells, which have an immature rather than a mature peripheral phenotype. No tumor development was observed in control littermates and transgenic mice expressing a truncated form of c-Rel. Tumor formation was correlated with the presence of constitutive p50/v-Rel DNA binding activity and overexpression of several kB regulated genes in v-*rel* transgenic thymocytes, however, v-Rel is also transforming in transgenic thymocytes lacking p50, indicating that p50/v-Rel heterodimer formation is not essential for the transforming activity of v-Rel. The transforming activity of v-Rel in p50 null mice mice has been identified as v-Rel/v-Rel homodimers. Since tumors represent immature T lymphocytes, constitutive v-Rel expression appears to be leukemogenic at earlier stages of T cell development.

Activation of the c-Rel transcription factor in chicken thymocytes undergoing apoptosis

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The chicken c-rel protooncogene encodes a transcription factor of the Rel/NF-κB family which is predominantly expressed in hematopoietic organs and in embryonic cells undergoing apoptosis. In this study, we focused on the role of c-rel in thymocyte apoptosis. The c-Rel protein is found in numerous cell types in an inactive form retained in the cytoplasm by ankyrin-repeat proteins of the IκB family (p40, p115 or p124). Upon stimulation, the inhibitory proteins are phosphorylated and degraded, thus releasing the transcription factor which may enter the nucleus and activate transcription.

Our work was performed on organotypic cultures of thymuses dissected from embryos at 19 days of development. Under these conditions, medullary thymocytes undergo massive apoptotic death within a few hours, whereas other cellular components of the thymus remain intact.

In situ hybridization on freshly dissected thymuses, before the onset of apoptosis, reveals that mRNAs of c-rel and its inhibitors p40 and p115 are predominantly expressed in antigen presenting (macrophages and dendritic cells) and stromal cells. Expression of the c-Rel protein (detected by immunocytochemistry) is restricted to the cytoplasm of these cells. In thymocytes on the other hand, although we observe a low but significant amount of the three mRNAs, no c-Rel protein can be detected. As apoptosis proceeds, an increasing number of medullary thymocytes express the c-Rel protein in their cytoplasm and some of them in their nucleus, while other cell types seem to be unaffected. Nuclear localization of c-Rel is correlated with the formation of condensing cells and apoptotic bodies. During this process, the level of the c-rel mRNAs remains unchanged, while the levels of p40 and p115 mRNAs dramatically decrease in apoptotic cells. Western blot analysis of nuclear and cytoplasmic extracts from these cells indicates that indeed c-Rel relocalizes into the nucleus as a result of the organotypic culture.

In conclusion, as medullary thymocytes undergo apoptosis, the expression of c-Rel is induced probably through a posttranscriptional mechanism since the level of c-rel mRNAs does not change. Moreover, the protein is translocated to the nucleus, thus becoming a functional transription factor, which might result from the observed decrease in the expression of the IκBs. The above events are not observed in other cellular components of the thymus that are not apoptotic. Therefore, these results suggest that the chicken c-rel protooncogene takes an active part in the mechanism of thymocyte apoptosis.

v-relER Transformed Bone Marrow Cells of Chicken: an *in vitro* Model System for Differentiation of Antigen-Presenting Dendritic Cells.

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Over the past years we have developed an *in vitro* system for differentiation of antigen-presenting dendritic cells. This system is based on the conditional, hormone-inducible oncoprotein v-RelER, which contains the v-rel oncogene fused to the hormone binding domain of the human estrogen receptor. In the presence of estrogen v-RelER is active and transforms hematopoietic progenitor cells present in chicken bone marrow *in vitro*. This allows to obtain clonal cell populations with a high proliferative capacity in culture. Cells transformed by v-rel have been assigned to different cell lineages according to surface marker expression and rearrangement of the immunoglobulin genes. T-lymphoid cells, cells at different stages of B cell development (including mature B cells), and myeloid cells are such targets for v-rel-specific transformation. In the v-relER system inactivation of v-RelER by addition of an estrogen antagonist enables cells to continue their normal differentiation program. We have developed conditions to achieve v-relER cell differentiation into dendritic cells, therefore identifying a progenitor for dendritic cells as another target for v-rel. It still remains open whether such cells can differentiate into other related hematopoietic cell types. Interestingly, v-relER progenitors grown with estrogen, exhibit some B-lymphoid properties, like the presence of surface IgM and IgA, or BSAP activity (B cell specific activator protein, in collaboration with P. Dörfler and M. Busslinger, IMP, Vienna, Austria). These lymphoid properties are downregulated upon induction of differentiation. However, v-relER cells do not produce immunoglobulin molecules or Ig-specific mRNA and have their Ig genes in germline configuration. Therefore, we speculate that v-relER cells express Fc receptors or related molecules that bind to soluble Ig molecules present in the chicken serum of the culture media. Fc receptor expression would be expected for dendritic cells.

We are also interested in exploring the gene expression repertoire of v-relER dendritic cells to gain insight into the cell biology and function of these professional antigen-presenting cells. As a first appproach, we have analysed the tyrosine kinase expression by domain-directed differential display. Several receptor and non-receptor tyrosine kinases were identified. Additionally, we are interested in determining how v-relER dendritic cells migrate and which stimuli attract them to lymphoid organs, since both activities are essential for antigen presentation. As a first step, we analysed v-relER dendritic cells for expression of cytoskeletal and cell adhesion molecules. These data will be presented.

The Role of NF-κB c-*rel* and RelA in Embryonic Limb Bud Development. Paul Bushdid, Fiona Yull, Mike Byrom, Haijing Li, Chih-Li Chen, and Lawrence Kerr. Vanderbilt University School of Medicine, Dept. of Microbiology and Immunology and Cell Biology, Nashville, TN 37232-2363 USA

The ubiquitous transcription factor, nuclear factor-kappaB (NF-κB), stimulates the expression of a number of genes involved in immune responses, acute phase reactions, and viral infections. The large array of growth factors, cytokines, and viral proteins which activate κB-enhancer binding activity suggests that the NF-κB family plays a pivotal role in the regulation of such cellular processes as proliferation, differentiation, and programmed cell death. Recent evidence from this laboratory suggest that tissue specific expression patterns for NF-κB factors throughout embryonic development may have an important physiological consequence in morphologic patterning in the developing embryo. We have defined the embryonic expression patterns of c-*rel* and RelA in both mouse and chicken model systems and have found both NF-κB factors are overexpressed during specific temporal windows during limb bud development. c-Rel mRNA is overexpressed in the developing progress zone and is not expressed in either the apical ectodermal ridge (AER) or the zone of polarizing activity (ZPA). Transgenic mice bearing a c-*rel* promoter/lacZ construct demonstrate expression of c-*rel* directed ß-galactosidase activity in the developing limb buds which disappear prior to birth. The appearance of c-*rel* in the limb bud correlates strongly with the expression of the homeodomain protein, Msx-1. We demonstrate that the Msx-1 promoter: (1) contains three functional κB-enhancer element as determined by mutagenesis studies; (2) is transcriptionally responsive to NF-κB activation; and (3) binds cRel *in vitro* and in extracts from limb bud mesenchyme. Surgical manipulation of avian limb buds demonstrate that removal of the AER abolishes c-*rel* expression in the developing limb suggesting that signal(s) emanating from the AER direct c-*rel* expression. Furthermore, implantation of beads bearing fibroblast growth factor-4 into AER minus limbs restores the expression of c-rel to the underlying mesenchyme. Current experiments are examining the role of the ZPA morphogens, *sonic hedgehog* (Shh) and retinoic acid on the expression pattern of c-*rel* in the developing limb. In addition retroviral vectors transferring NF-κB dominant negative mutants address the necessity of NF-κB activation for proper limb bud development.

Session : 3

Chairperson: David Baltimore

THE NF-κB/REL/IκB PARADIGM, D. Van Antwerp, K. Tashiro, M. Pando, E. Schwarz, and I. M. Verma, The Salk Institute, La Jolla, CA 92037. The mechanism by which the signal is transduced from the cell membranes to the nucleus through the cytoplasm is not fully understood, but many players in this cascade have recently been identified. The products of many of the rapidly induced early response genes are transcription factors which can "switch-on" other genes eventually leading to cell growth, differentiation and development. One family of transcription factors, NF-κB/Rel proteins are uniquely sequestered in the cytoplasm in association with an inhibitory protein, IκB. Upon stimulation with a wide variety of agents, IκB protein is modified and degraded, releasing NF-κB proteins which translocate to the nucleus and bind to decameric κB-binding sites. The activity of NF-κB proteins is therefore controlled by IκB protein. We have shown that upon addition of TNF-α to HeLa cells, IκB protein is phosphorylated, followed by rapid degradation. In the presence of inhibitors of degradation of IκB, phosphorylated IκB can be found associated with p65 (RelA) a member of the NF-κB binding proteins. We conclude that the phosphorylated form of IκB remains associated with NF-κB complex and degradation occurs *in situ* in the complex. We have localized the region and some specific sites of phosphorylation in IκB. Removal of C-terminal 39 a.a. (containing PEST sequences) generated a form of IκB which weakly associates with NF-κB but does undergo degradation. The mutated IκB also prevents transcriptional transactivation by NF-κB proteins. The signal induced degradation of IκBα does not require PEST sequences; however, loss of PEST sequences increases half-life of free IκBα.

We also report that in a variety of cells where NF-κB activation is compromised by transdominant IκBα, addition of TNFα causes apoptosis. Ordinarily, TNFα leads to NF-κB activation and subsequent activation of genes involved in immune responses. Thus, NF-κB proteins suppress signals for cell death. Finally, we will also discuss the association of NF-κB/Rel/IκB proteins with other cellular proteins.

Supported by NIH and ACS

NF-κB signalling mechanisms and novel protein associations.

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NF-κB release can occur by signal-dependent degradation or by basal turnover of its inhibitor IκBα. Given the demonstrations of a ubiquitin-proteasome pathway for signal-dependent IκBα degradation (1), that were mainly based on biochemical assays, and the lack of understanding the mechanism of basal turnover, we have made use of a tagged substrate/tagged ubiquitin assay (2) that allows investigation of signal-dependent and -independent degradation in intact cells. *In vivo*, tumor necrosis factor alpha (TNFα) induced breakdown of IκBα correlates with ubiquitin-conjugation within 5 min., strictly depending on the presence of the amino-terminal 70 amino acids. When the cells were left unstimulated no ubiquitinated IκBα species were detectable. In contrast, the protein domains required for basal turnover of IκBα differ from those that are fundamental for TNFα triggered degradation. Neither the amino-terminal SRD nor the carboxy-terminal PEST sequence are needed for basal turnover that essentially depends only on the core ankyrin repeat domain . Basal degradation is controlled by a mechanism independent of ubiquitin-conjugation since an IκBα mutant with all lysine residues changed to arginines, that still exhibited unperturbed p65 binding, has the same high turnover as the wild type molecule. Furthermore, despite its high turnover, IκBα is not ubiquitinated in unstimulated cells, when compared to c-Jun, whose basal turnover is accompanied by high ubiquitination of the molecule. Notwithstanding the differences in the requirements of protein domains and ubiquitin-conjugation for both degradation pathways, each one is significantly blocked by the proteasome- and calpain-inhibitor ALLN. It can be concluded that inherently different processes regulate signal-dependent and -independent degradation of IκBα. The potential role of structural determinants for signal-dependent degradation will be discussed.

The analysis of cellular cytosolic and nuclear NF-κB/IκB complexes by immunoprecipitation (3) and gradient centrifugation has revealed the presence of expected as well as additional novel protein complexes. By screening of expression libraries with recombinant NF-κB-p52 as a probe we have isolated "nuclear factor related kappa B" (RκB) as a transcription factor that binds with high affinity to a subgroup of NF-κB/Rel proteins, but

not to IκB proteins. RκB reveals interesting properties, as novel DNA binding and dimerization domains, as well as an inhibitory domain with repetitive sequences. The fact that RκB binds to NF-κB binding sites (4) and to cellular NF-κB-proteins suggests a role in NF-κB signal transduction.

References:

1.) Chen, Z., Hagler, J., Palombello, V.J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.* 9, 1586-1597.
2.) Treier, M., Staszewski, L.M., and Bohmann, D. (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. *Cell* 78, 1994
3.) Naumann, M., and Scheidereit, C. (1994) "Activation of NF-κB *in vivo* is regulated by multiple phosphorylations", *EMBO J.* 13, 4597-4607.
4.) Adams, B.S., Leung, K., Hanley, E.W. and Nabel, G.J. (1991). Cloning of RkB, a novel DNA-binding protein that recognizes the interleukin-2 receptor a chain kB site. *The New Biologist* 3, 1063-1073.

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Regulation of NF-κB by IκB-β

Stimulation with inducers that cause persistent activation of NF-κB results in the degradation of the NF-κB inhibitors, IκB-α and IκB-β. Despite the rapid resynthesis and accumulation of IκB-α, NF-κB remains induced under these conditions. We now report that IκB-β is also resynthesized in stimulated cells and appears as an unphosphorylated protein. The unphosphorylated IκB-β forms a stable complex with NF-κB in the cytosol, however this binding fails to mask the nuclear localizing signal and DNA binding domain on NF-κB. It appears therefore that during prolonged stimulation, IκB-β functions as a chaperone for NF-κB by protecting it from IκB-α and allowing it to be transported to the nucleus.

Interaction of Various IκB Proteins With NF-κB.

Nancy Rice. ABL-Basic Research Program, NCI- Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201 USA

With the isolation of IκBα by Simon Whiteside and Alain Israel, there are now three major inhibitors of NF-κB complexes: IκBα, IκBβ, and IκBε. I will describe efforts to compare their properties with respect to turnover, inducibility, and dimer preference. I will also describe my efforts at characterizing IκBε, as part of a collaborative study with Drs. Whiteside and Israel. Briefly, IκBε is associated with NF-κB dimers in all cells tested to date, and in most cells it binds significantly more NF-κB than does IκBα. IκBε is constitutively phosphorylated and is degraded following exposure of B cells or monocytes to LPS, but not to PMA. Degradation is blocked by the proteasome inhibitor ALLN. I will also describe studies on the interaction of IκBα with homodimers of p50, p65, and c-Rel. We have made a large number of mutants of these proteins and have followed the effect of the mutations on ability to bind IκBα. This has allowed us to deduce which specific regions are important for binding.

THE 90 kDa RIBOSOMAL S6 KINASE (pp90^{rsk}) INDUCIBLY PHOSPHORYLATES IκBα AND STIMULATES ITS DEGRADATION *IN VITRO*. Lucy Ghoda[1], Xin Lin[2] and Warner C. Greene[2,3] Dept. of Pharmacology[1], University of Colorado Health Sciences Center, Denver, Colorado 80262 and Gladstone Institute of Virology & Immunology[2], and Department of Medicine and Microbiology and Immunology[3], University of California, San Francisco, CA 94110

Nuclear factor κB (NF-κB) is a transcription factor that is negatively regulated by its physical association with a family of cytoplasmic ankyrin rich inhibitors including IκBα. To activate NF-κB nuclear translocation and function, IκBα must be phosphorylated, conjugated to ubiquitin, and degraded by the 26S proteasome complex. At present, the exact identity of the IκBα kinase(s) remains elusive. We have recently focused our studies on the potential role of pp90^{rsk} as an IκBα kinase. This enzyme lies downstream of MAP kinase in the ras-raf-MEK-MAPK pathway induced by phorbol ester (PMA) and various growth factors. We have found that PMA induces pp90^{rsk} phosphorylation of IκBα and that this phosphorylation is dependent on the key N-terminal regulatory serine residues located at positions 32 and 36 in the native IκBα protein. The analysis of single serine substitution mutants of IκBα has revealed that serine 32 is the principal site of phosphorylation by activated pp90^{rsk}. This finding raises the possibility that different kinases may mediate phosphorylation at serine 32 and 36. N-terminal phosphorylation of IκBα by pp90^{rsk} appears functionally important as it leads to the rapid degradation of IκBα *in vitro*. Analysis of cotransfected COS cells has further shown that pp90^{rsk} and IκBα are physically associated *in vivo*. Finally, we have found that pp90^{rsk} kinase activity is stimulated by phorbol ester but not by HTLV-1 Tax or TNF-α, each of which are potent inducers of NF-κB. We conclude that pp90^{rsk} likely corresponds to one of perhaps multiple intracellular IκBα kinases that differentially mediate phosphorylation of IκBα in a signal specific manner. Supported by 5RO1 GM49055, American Cancer Society Research Award, VM-91, a Junior Faculty Research Award of the American Cancer Society to L. Ghoda.

BIOCHEMICAL CHARACTERIZATION AND PURIFICATION OF THE IκB KINASE. F.Mercurio,* J. DiDonato,# J. Li,* D. Young,* M. Karin,# and M. Barbosa*. Signal Pharmaceuticals Inc*. San Diego, CA 92121 and UC San Diego#, La Jolla, Ca 92037.

NF-κB plays a pivotal role in the highly specific pattern of gene expression found for immune, inflammatory and acute phase response genes, including interleukin 1 (IL-1), interleukin 8, tumor necrosis factor (TNF) and certain cell adhesion molecules. The IκB family of proteins are important regulators of NF-κB activity, wherein IκB modulates cytoplasmic to nuclear translocation associated with NF-κB activation *in vivo*. Stimulus-induced phosphorylation of IκB render it a target for ubiquitination and subsequent degradation by the proteosome. In an effort to better elucidate the mechanism of NF-κB activation and the nature of the IκB kinase(s) we initiated studies to biochemically characterize the inducible phosphorylation sites on IκBα and IκBβ. Serines 32 and 36 of IκBα were phosphorylated in response to TNFα and IL-1. Substitution of either serine blocked or slowed stimulus-induced IκBα degradation. Moreover, substitution of homologous sites on IκBβ, serines 19 and 23, also inhibit inducible IκBβ degradation. We developed an assay to identify the kinase responsible for inducible phosphorylation of IκBα/β at these sites. Incorporation of the IκBα/β kinase assay with classical biochemical fractionation and a novel affinity chromatography step has enabled us to purify the IκB kinase. We believe that activation of the IκB kinase is the first critical step in NF-κB activation. Identification of the IκB kinase will allow us to develop potent anti-inflammatory and immunosuppressive drugs which selectively block NF-κB activation and NF-κB responsive genes.

Characterisation Of I kappa B Epsilon, A Novel Member Of The I kappa B Family Of Proteins.

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In a search for novel proteins capable of interacting with the transcription factor NF-κB, we performed a yeast 2-hybrid screen using as bait, the p52 protein fused to the DNA-binding domain of GAL4. Out of 250,000 independent clones screened, we isolated cDNAs encoding three novel proteins. Further characterisation revealed that one of these cDNAs encodes a novel member of the IκB family of proteins, which we have named IκBε. Northern blot anaysis reveals that IκBe is encoded by a 2.2kb message that is ubiquitously expressed, and whose levels are induced during differentiation of HL60 cells into either monocytes or neutrophils. IκBε consists of two isoforms: a 38kD protein that contains 6 ankyrin repeats as well as two serine residues whose sequence context strongly resembles that of serines 32 and 36 of IκBα, and a 55kD proetein that contains an additional N-terminal domain. Co-precipitation studies demonstrate that these proteins can interact with Rel proteins in vitro and in vivo. Furthermore, they are capable of inhibiting both NF-κB binding in vitro as well as affecting NF-κB-mediated transactivation in vivo. The specificity of binding of the two isoforms to various Rel proteins and their various effects upon NFκB-mediated transactivation in vivo will be presented. The possible role of the two different isoforms of IκBε in the regulation of NFκB activity by extracellular stimuli and differentiation will be discussed.

Session 4:

Chairperson: Warner C. Greene

Activation of NF-κB by Two Novel Pathways

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The ER Overload Pathway

The endoplasmic reticulum (ER) is an organelle that can provide signals to the cell nucleus that change gene expression. One pathway is induced by the accumulation of mal-folded luminal and membrane-bound secretory proteins. This leads to the activation of ER membrane-bound kinases that phosphorylate unknown messenger protein(s). These activate a nuclear transcription factor called UPRF which, upon binding to glucose response elements, turns on genes. The products of these genes, such as BIP or PDI, end up in the ER lumen where they help in folding the malfolded proteins that orignally provided the gene-inductory stimulus.

A second pathway is induced by a change in the lipid composition of the ER membrane. When its content of sterol drops an ER-resident transcription factor precursor is cleaved by a sterol-regulated protease. The released SREBP factor, a basic helix-loop-helix protein, translocates to the nucleus where it induces genes whose products are involved in cholesterol biosynthesis (HMGCoA synthase) or uptake (LDL receptor).

We have found that NF-kB plays a role in a third, novel ER-nuclear signal transduction pathway. This came from the observation that a number of drugs known to perturb the organelle's function could induce activation of the transcription factor. Among these drugs were glycosylation inhibitors, such as tunicamycin and 2-deoxyglucose, and transport inhibitors, such as brefeldin A and monensin. Likewise, the over- or atopic expression of seceretory membrane proteins, such as μ heavy chain, MHC class I, influenca hemagglutinin or a truncated Hepatitis B virus surface antigen, could potently activate NF-kB. This suggested that the mere accumulation of (correctly folded) seceretory membrane proteins in the ER provides a novel signal leading to NF-kB activation. This signal was studied by using the ER-resident Adenovirus protein E3/19K as a model system. The wildtype protein turned out as a potent novel inducer of NF-kB when overexpressed at very low levels. In contrast, a variety of mutant forms that were no longer retained in the ER but transported to the cell surface were deficient of activating NF-kB even at high expression levels. From these findings, we concluded that the signal was just the overload of the compartment with membrane protein.

We reasoned that an increase in the protein/lipid ratio in the ER may perturb the organelle's function of sequestering calcium. Leakage of the cation into the cytosol could provide a stress signal activating NF-kB. This hypothesis was supported by our observation that two structurally unrelated intracellular calcium chelators, TMB-8 and BAPTA-AM, potently prevented NF-kB activation by E3/19K and various ER drugs, but not the activation of NF-kB by TNF. Further support for the involvement of calcium came from the finding that two structurally unrelated inhibitors of the ER-resident Ca-ATPase, thapsigargin and cyclopiazonic acid, rapidly induced the factor. The increased cytosolic calcium appeared to trigger an increase in reactive oxygen (ROIs) production, as was measured by FACS using a peroxide sensitive dye. This secondary signal was also

important as many antioxidant compounds prevented NF-kB activation in response to ER stress signals.

The ER overload pathway was pharmacologically distinct from the unfolded protein response pathway. The drug castanospermine was found to potently activate BIB expression but not NF-kB activation. Likewise, none of the classical NF-kB inducers, such as TNF or PMA, was found to induce genes activated by the unfolded protein response.

We have shown that the ER can respond to a sudden increase in membrane proteins --as would occur upon an acute viral infection of the cell-- by the activation of NF-kB, a pro-inflammatory genetic switch. An apealing aspect of this primitive antiviral response is its broad specificity: it can be elicited by a wide variety of distinct viral membrane proteins from various classes of viruses.

A Tyrosine Kinase Pathway

Pervanadate (PV) is a potent inducer of T cell activation by virtue of activating tyrosine kinases through inhibition of tyrosine protein phosphatases (mainly CD45). PV is also a potent activator of NF-kB in T cell lines. This prompted us to investigate whether tyrosine phosphorylation is directly involved in NF-kB activation.

Upon PV treatment of T cells, IkB-alpha was found to be stoichiometrically phosphorylated on tyrosine 42 in its regulatory N-terminus. This event physically released IkB-alpha from NF-kB without a subsequent degradation of the inhibitor. Compared to the well-studied phosphorylation of IkB-alpha on serines 32 and 36, tyrosine phosphorylation of IkB-alpha was thus novel in two ways: it allowed dissociation of the NF-kB--IkB complex and it did not trigger ubiquitin conjugation and subsequent degradation of IkB. We suspect that both properties relied on the binding of an SH2-domain containing protein to tyrosine-phosphorylated IkB.

A physiological stimulus leading to tyrosine phosphorylation was reoxygenation of hypoxic cells. This condition which is of considerable pathophysiological importance is known to lead to a pro-inflammatory genomic response.

Selected Recent References by Topics

A Novel Pathway Between ER and Nucleus

Pahl, H. and P.A. Baeuerle (1995). Influenza hemagglutinin activates transcription factor NF-kB. *J. Virol.* **69**: 1480-1484.

Pahl, H.L. and P.A. Baeuerle (1995). A novel signal transduction pathway from the endoplasmatic reticulum to the nucleus is mediated by transcription factor NF-kB. *EMBO J.*, **14**: 2580-2588.

Pahl, H.L., M. Sester, H.-G. Burgert and P.A. Baeuerle. (1996) Activation of transcription factor NF-kB by the adenovirus protein E3/19K requires its ER retention. *J. Cell. Biol.* **132**: 511-522.

Pahl, H.L. and P.A. Baeuerle. ER stress-mediated NF-kB activation requires both Ca^{2+} and reactive oxygen intermediates as second messengers. submitted

IkB-a Phosphorylation and Proteolysis

Traenckner, E.B.-M., S. Wilk and P.A. Baeuerle (1994). A proteasome inhibitor prevents activation of NF-kB and stabilizes a newly phosphorylated form of IkB-a that is still bound to NF-kB. *EMBO J.* **13**: 5433-5441.

Traenckner, E.B.-M., H.L. Pahl, K.N. Schmidt, S. Wilk and P.A. Baeuerle (1995). Phosphorylation of human IkB-a on serines 32 and 36 controls IkB-a proteolysis and NF-kB activation in response to diverse stimuli. *EMBO J.* **14**: 2876-2883.

Traenckner, E.B.-M. and P.A. Baeuerle (1995). Appearance of apparently ubiquitin-conjugated forms of IkB-a during its phosphorylation-induced degradation in intact cells. *J. Cell Sci.*, Suppl. **19**: 79-84.

Imbert, V., R.A. Rupec, P. Auberger, H.L. Pahl, E. B.-M. Traenckner, C. Müller-Dieckmann, D. Farahifar, B. Rossi, P.A.Baeuerle and J.-F. Peyron. Tyrsoine phosphorylation of IkB-a activates NF-kB without proteolytic degradation of IkB-a, submitted.

ROIs and Gene Activation

Schmidt, K.N., P. Amstad, P. Cerutti and P.A. Baeuerle (1995). The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kB.*Chem.& Biol.* 2: 13-22.

Rupec, R.A. and P.A. Baeuerle (1995). The genomic response of tumor cells to hypoxia and reoxygenation: differential activation of transcripton factors AP-1 and NF-kB. *Eur. J. Biochem.* **234**: 632-640.

Los, M., W. Dröge, K. Stricker, P.A. Baeuerle and K. Schulze-Osthoff (1995). Hydrogen peroxide as a potent activator of T lymphocyte functions. *Eur. J. Immunol.* **25**: 159-165.

Los, M., H. Schenk, K. Hexel, P.A. Baeuerle, W. Dröge and K. Schulze-Osthoff (1995). IL-2 gene expression and NF-kB activation through CD28 requires reactive oxygen production by 5-lipoxygenase. *EMBO J.* **14**: 3731-3740.

Munroe, D.G., E.Y. Wang, J.P. MacIntyre, S.S.C. Tam, D.H.S. Lee, G.R. Taylor, L. Zhou, R.K. Plante, S.M.I, Kazmi, P.A. Baeuerle and C.Y. Lau (1995). Novel intracellular signaling function of prostaglandin H synthase-1 in NF-kB activation. *J. Inflamm.* **45**: 260-268.

Schmidt, K.N., E. B.-M. Traenckner, B. Meier and P.A. Baeuerle (1995). Induction of oxidative stress by okadaic acid is required for activation of transcription factor NF-kB. *J. Biol. Chem.* **270**: 27136-27142.

Schmidt, K.N., M. Podda, L. Packer and P.A. Baeuerle (1996). The anti-psoriatic drug anthralin activates transcription factor NF-kB in keratinocytes. *J. Immunol.*, in press.

NF-kB in the Brain

Kaltschmidt, C, B. Kaltschmidt, and P.A. Baeuerle (1993) Brain synapses contain inducible forms of the transcription factor NF-kB. Mech. Dev. 43: 135-147.

Kaltschmidt, C., B. Kaltschmidt, T. Henkel, H. Stockinger and P.A. Baeuerle (1995). Selective recognition of the activated form of transcription factor NF-kB by a monoclonal antibody. *Biol. Chem. Hoppe-Seyler* **376**: 9-16.

Kaltschmidt, C., B. Kaltschmidt, H. Neumann, H. Wekerle and P.A. Baeuerle (1994) Constitutive NF-kB activity in neurons. *Mol. Cell. Biol.* **14**: 3981-3992.

Kaltschmidt, C., B. Kaltschmidt and P.A. Baeuerle (1995). Stimulation of ionotropic glutamate receptors activates transcription factor NF-kB in primary neurons. *Proc. Natl. Acad. Sci. USA* **92**: 9618-9622.

Carter, B.D., C. Kaltschmidt, B. Kaltschmidt, N. Offenhäuser, R. Böhm-Mattaei, P.A. Baeuerle and Y.-A. Barde. Selective activation of NF-kB by nerve growth factor through the neurotrophin receptor p75. *Science* **272**:542-545.

Kaltschmidt, C., R. Linker, B. Kaltschmidt, and P.A. Baeuerle. Expression of cyclooxygenase-2 in neurons is dependent on constitutively active NF-kB. in preparation

NF-kB in Disease

Kaltschmidt, C, B. Kaltschmidt, J. Lannes-Vieira, G. Kreuzberg, H. Wekerle, P.A. Baeuerle and J. Gehrmann (1994). Activation of transcription factor NF-kB in microglia during experimental autoimmune encephalomyelitis. J. *Neuroimmunol.* **55**: 99-106.

Marok, R., P.G. Winyard, A. Coumbe, M.L. Kus, K. Gaffney, S. Blades, P.I. Mapp, C.J. Morris, D.R. Blake, C. Kaltschmidt and P.A. Baeuerle (1996). Activation of transcription factor NF-kB in human inflamed synovial tissue. *Arthritis & Rheumatism* **39**: in press.

Brand, K., G. Rogler, A. Bartsch, R. Knuechel, M. Page, C. Kaltschmidt, P.A. Baeuerle and D. Neumeier. Transcription factor NF-kB/Rel is activated in the atherosclerotic lesion. *J. Clin. Invest.*, in press.

Brand, K., S. Page, A.K. Walli, D. Neumeier and P.A. Baeuerle (1996). Role of NF-kB in atherogenesis. *Experimental Physiol.*, in press.

Brand, K., T. Eisele, U. Kreusel, M. Page, S. Page, M. Haas, A. Gerling, C. Kaltschmidt, F.-J. Neumann, P.A. Baeuerle, A.K. Walli and D. Neumeier. Dysregulation of monocytic nuclear factor kappaB by oxidized low density lipoprotein. submitted.

Kaltschmidt, C., B. Kaltschmidt, B. Volk and P.A. Baeuerle. Transcription factor NF-kB is activated in primary neurons by amyloid bA4 and in neurons surrounding early plaques from Alzheimer disease patients. submitted.

NF-kB and Apoptosis

Grimm, S., M. Bauer, P.A. Baeuerle and K. Schulze-Osthoff (1996). Bcl-2 downregulates the activity of transcription factor NF-kB which is induced upon apoptosis. *J. Cell. Biol.*, in press.

Schmitz, M.L., A. Indorf, F.P. Limbourg, H. Städtler, E. B.-M. Traenckner and P.A. Baeuerle (1996). The dual effect of adenovirus 5 E1A 13S protein on activation of transcription factor NF-kB is antagonized by E1B 19 kDa. *Mol. Cell. Biol.*, in press.

Limbourg, F.P., H. Staedler, G. Chinnadurai, P.A. Baeuerle and L. Schmitz (1996). A hydrophobic region within the Adenovirus E1B 19 kDa protein is necessary for the transient inhibition of NF-kB activated by different stimuli. *J. Biol. Chem.*, in press.

Control of NF-κB activity by IκB molecules

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The transcription factor NF-κB is neutralized in non-stimulated cells through cytoplasmic retention by IκB inhibitors. In mammalian cells, two major forms of IκB proteins, IκBα and IκBβ, have been identified. Upon treatment with a large variety of inducers, IκBα and IκBβ are proteolytically degraded, resulting in NF-κB translocation into the nucleus. Recent observations suggest that phosphorylation of Serines 32 and 36, and subsequent ubiquitination of Lysine 21 and 22 of IκBα, control its signal-induced degradation. I will present the results of recent experiments addressing the regulation of the activity of IκB molecules :

- we have demonstrated that critical residues in the N-terminal region of IκBβ (Serines 19 and 23) as well as its C-terminal PEST region, control IκBβ proteolysis. However Lys 9, the unique Lysine residue in the N-terminal region of IκBβ, is not absolutely required for its degradation. We also demonstrate that following stimulation, an underphosphorylated non-degradable form of IκBβ accumulates. Surprisingly, our data suggest that, unlike IκBα, the state of phosphorylation of IκBβ does not change after stimulation, suggesting that degradation requires another inducible step.

We have identified hyperosmotic shock as a new NF-κB-activating signal. Interestingly, in 70Z/3 cells, hyperosmotic shock induces the degradation of IκBα through a pathway which is independent of proteasomes, does not require Ser 32 and 36 nor the C-terminal PEST-region, and is resistant to antioxidants.

- We have characterized a mutant cell line which is resistant to most NF-κB activating stimuli. Degradation of the 2 known IκB molecules (as well as phosphorylation of IκBα) does not take place in response to PMA, LPS or IL1. Measurement of the redox level of the mutant cell line does not show any significant difference with its wt counterpart. However phosphatase inhibitors like calyculin A, as well as hyperosmotic shock, do activate NF-κB in the mutant line.

Based on these data, I will discuss the similarities as well as differences in the mechanisms leading to degradation of the 2 IκB molecules.

- in a 2-hybrid screen, Simon Whiteside has isolated (and will present in a separate talk) and characterized in collaboration with Nancy Rice, a new

IκB molecule, IκBε. Like α and β, IκBε has several ankyrin repeats, a putative C-terminal PEST domain and 2 serines in the N-terminal region in an environment similar to that found in the 2 other inhibitors (and in Drosophila cactus). The cDNA exhibits 2 potential ATG start sites, of which only the second one seems to be used. It is mostly associated with non-p50/p52 containing complexes, and is degraded with slow kinetics in response to classical NF-κB-activating stimuli. In several cell types, IκBβ and IκBε represent the major p65 and c-rel-associated inhibitors.

NF-κB activity in transgenic mice : developmental regulation and tissue specificity

In order to be able to follow the pattern of NF-κB activity during mouse development and in the adult following various stimulations, we have generated transgenic mice carrying two LacZ reporter constructs, each driven by promoter elements which are dependent on the presence of nuclear NF-κB/Rel activity. The analysis of these mice did not identify nuclear NF-κB/Rel activity in early development prior to implantation nor during the gastrulation processes. Earliest expression of the LacZ transgene was detected on day E12.5 in the nervous system. Before birth LacZ expression was seen in discrete regions of the rhombencephalon of the developing brain, in the spinal medulla, in some of the blood vessels, and in the thymus. After birth the NF-κB/Rel activity in the thymus remained but nuclear activity was also found in the the bone marrow, in the spleen and in the capsule of the lymph nodes. In the central nervous system drastic changes in NF-κB/Rel activity could be observed in the first 3 weeks after birth, when the cortex and the cerebellum reach functional and morphological maturity. Considering the results of the p50, p65, relB and c-rel knock-out mice and our present findings we believe that the NF-κB/Rel proteins known so far are probably not implicated in processes of early development and differentiation of the different tissues, but rather in maintaining their function once matured.

SIGNAL TRANSDUCTION FROM TNFα RECEPTORS TO NFκB

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Tumor Necrosis Factor (TNFα) is a pleitropic cytokine whose function is mediated through two distinct receptors designated p60 (also referred as type I or p55) and p80 (also referred as type II or p75). The p60 receptor is expressed in almost all cell types whereas the p80 receptor is expressed primarily in endothelial cells and cells of the immune system. Both p60 and p80 belong to the growing TNF and NGF receptor superfamily which includes Fas, CD27, CD30, CD40 and OX40 (1), which are characterized by cysteine rich domains in the extracelular portion of the receptor. The stucture of the cytoplasmic domains of these receptors are quite different, which might be at the origin of the different responses of cells to TNFα. It is particularly interesting that TNFα can trigger cytotoxic effects on some cells while it induces cell proliferation in others.

Several proteins have been identified that interact with TNF receptor superfamily to initiate intracellular signal transduction. These proteins fall into two structural classes, containing either death domains (2) or TRAF domains (3,4). TRADD, a death domain protein, interacts with p60 receptor in a ligand dependent manner to initiate signaling cascades leading to apoptosis and activation of NFκB. The C terminal region of p80 receptor constitutively interacts with TRAF1 and TRAF2. also leading to NFκB activation. In addition to the TRAF domain in the C terminal region,TRAF proteins contain a RING finger domain in the N terminal region which appears essential for TRAF2 mediated NFκB activation. However, TRAF2b, a protein containing an altered RING finger region due to a 7 amino acid insertion is still capable of mediating NFκB activation. TRAF2b has a tissue specific distribution but its significance is still unkown. How TRADD and TRAF proteins are able to transmit signals downstream remains to be known.

Downstream signaling from TNFα receptors has been studied in a variety of systems. Phospholipases, sphingomyelinases, protein kinases, protein phosphatases and superoxide radicals have been implicated in TNFα-mediated cell killing and mitogenicity.(5). The activation of ceramide activated protein kinase, protein kinase C, Raf kinase, MAP kinase and Jun kinase by TNFα have been reported. In addition, two separate ser/thr TNFα receptor-associated protein kinases (p60TRAK and p80TRAK) have been identified(6,7). Whether all these kinases are implicated in the mitogenic or the cytotoxic response to TNFα is unknown, but it is likely that different protein kinases are activated in differerent cells. Thus, phorbol esters can induce activation of NFκB in MCF7 cells but have no effect in NIH 3T3 or WEHI 164 cells.

TNFα has been shown to mediate its action through activation of NFκB and other transcriptional factors, including c-fos, c-jun, c-myc and egr-1. Although all these are genes implicated in growth regulation, these transcription factors are activated in cells in which TNFα has cytotoxic

effects, indicating that the signals leading to cell toxicity are induced independently or even mediated by mitogenic signals. We have studied TNFα-induced activation of NFκB in WEHI 164 cells (a mouse fibrosarcoma to which TNFα is cytotoxic) and in NIH3T3 in which TNFα induce mitogenic signals. In both cases, TNFα induces a rapid and transient activation of NFκB which is followed by a second phase in which NFκB complex remains in the nucleus at about 50% of maximum(8). A persistent nuclear localization of NFκB appears a requirement for the cytotoxic effect to take place. NFκB is a family of dimers composed by the Rel/NFκB family of proteins (9). Dimers have different binding specificity to DNA and can therefore have differential transcriptional activity. If the turn on of transcriptional programs can influence the final response to TNFα, it is of interest to know the composition of NFκB complexes. In WEHI 164 cells, which are killed by TNFα under any circumstance, p50, p65, p52 and RelB can be detected in active NFκB complexes. In NIH3T3 cells, in which TNFα can induce proliferative signals, only p50 and p65 are the components of active NFκB complexes.

References.

1. Smith, C.A., Farrah, T. and Goodwin, R.G.. (1994). Cell **76**, 959-962.
2. Hsu, H., Xiong, J. and Goeddel, D.V. (1995). Cell **81**, 495-504.
3 Rothe, M., Wong, S.C., Henzel, W.J. and Goeddel, D.V. (1994). Cell **78**, 681-692.
4. Rothe, M., Sarma, V., Dixit, V.M. and Goeddel, D.V. (1995). Science **269**, 1424-1427.
5. Deyaert, R. and Fiers, W. (1994). FEBS Letters **340**, 9-18.
6. Darnay, R., Reddy, S.A. and Aggarwal, B.B. (1994). J. Biol. Chem. **269**, 20299-20304.
7..Darnay, R., Reddy, S.A. and Aggarwal, B.B. (1994). J. Biol. Chem. **269**, 19687-19690.
8.Claudio, E., Segade, F., Wrobel, K., Ramos, S., Bravo, R. and Lazo. P.S.(1996). Exp. Cell Research **224**, 63-71.
9. Baeuerle, P.A. and Henkel, T. (1994). Ann. Rev. Immunol. 12, 141-179.

RHO, CDC42 AND RAC PROTEINS ACTIVATE THE NUCLEAR FACTOR κB BY A RAS- AND RAF-INDEPENDENT PATHWAY

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The family of Rho proteins, including Rho A, Rho B, Rho C, Rho G, CDC42, Rac 1, Rac 2, and TC10, are members of the Ras superfamily of GTPases . Rho proteins have been related to the regulation of multiple cellular functions, including transformation , apoptosis , metastasis and cytoskeletal organisation . Rho proteins regulate the activation of JNK/SAPKs, a family of intracellular kinases which are part of the internal cascades leading to the nucleus through modulation of the *jun* transcription factor. Furthermore, Rho proteins also regulate the c-*fos* serum response element (SRE) by a still unknown mechanism and are involved in the regulation of relevant enzymes such as PLA2, PLD, PI3K and PI5K as well as intracellular PAK kinases.

We report here a novel signalling pathway activated by the Rho proteins which may be responsible for their biological activities. The human Rho A, CDC42 and Rac 1 proteins efficiently induce the transcriptional activity of nuclear factor κB (NF-κB). This activation is achieved by targetting the inhibitory protein IkBα but not IkBβ or p105. The activation of NFκB is not species specific since all three genes induce activation of NFκB in Cos-7, NIH3T3 and Jurkat cells. The mechanism of κB activation is independent of the Ras GTPase and the Raf-1 kinase. Physiological activation of NF-κB by TNFα depends upon CDC42 and Rho A, but not Rac 1 proteins, since this activity is drastically inhibited by their respective dominant negative mutants. Thus, members of the Rho family of GTPases are involved in the regulation of NF-κB-dependent transcription.

1.- Perona R., Esteve P., Jimenez B., Ballestero R.P., and Lacal J.C. (1993). *Oncogene* 8, 1285-1292.

2.- Jiménez, B., Arends M., Esteve P., Perona R., Sanchez R., Ramón y Cajal S., Wyllie A. and Lacal J.C. (1995). *Oncogene* 10, 811-816.

3.- Esteve, P. , del Peso, L. and Lacal, J.C. (1995) *Oncogene* 11, 2657-2665.

Tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α

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The transcription factor NF-κB regulates expression of many genes participating in immune and inflammatory responses. In T lymphocytes, NF-κB is sequestered in the cytosol by the inhibitor IκB-α. De novo phosphorylation of IκB-α on serine residues induces its ubiquitin-dependent degradation. Here, we report an alternative mechanism of NF-κB activation. When Jurkat T cells were stimulated with the protein tyrosine phosphatase inhibitor and potent T cell activator pervanadate, IκB-α was rapidly and stoichiometrically phosphorylated on tyrosine. This modification did not cause proteolytic degradation of IκB-α but nevertheless dissociated IκB-α from NF-κB thereby inducing nuclear NF-κB DNA-binding activity. Jurkat mutant cells, deficient for expression of the T-cell specific tyrosine kinase $p56^{lck}$ showed a defect in both pervanadate-induced IκB-α phosphorylation and NF-κB activation. Reoxygenation of hypoxic cells was found to be a physiological condition stimulating IκB-α tyrosine phosphorylation. Inducible tyrosine phosphorylation of IκB-α thus represents an alternative, proteolysis-independent mechanism of NF-κB activation which may directly couple NF-κB to receptor-associated tyrosine kinases.

Session: 5

Chairperson: Patrick A. Baeuerle

Molecular Regulation of the T Lymphocyte Responses in the Healthy and Diseased Immune System.

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T lymphocytes can undergo either an activation/proliferation response including the induction of NF-κB or a programmed death response following T cell receptor engagement. The choice between these outcomes is dictated by the activation state of the T lymphocyte as well as the concentration of antigen to which the T cell is exposed. We have found that distinct signaling components of the multi-chain T cell receptor govern either the activation or death responses. Moreover, receptors in the TNF receptor superfamily may participate in both the induction of NF-κB and the activation of a unique class of cysteine proteases whose activity leads to apoptosis of the cell. These pathways appears to have multiple points of regulation. Genetic defects in the molecules of the lymphocyte death pathway in man lead to a syndrome of autoimmunity and dysregulated lymphocyte homeostasis.

References:

Kang, S-M., Tran, A-C., Grilli, M., and Lenardo, M.J.: NF-κB subunit regulation in nontransformed CD4+ T lymphocytes. *Science*, Vol. 256: 1452-1456, 1992.

Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middelton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.: Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome (ALPS). *Cell*. 81: 935-946, 1995.

Zheng, L., Fisher, G., Miller, R.E., Peschon, J., Lynch, D.H., and Lenardo, M.J.: Mature T lymphocyte apoptosis by tumor necrosis factor. *Nature*, 377: 348-351, 1995.

Combadiere, B., Freedman, M., Chen, L., Shores, E.W., Love, P., and Lenardo, M.J.: Qualitative and quantitative contributions of the T cell receptor ζ chain to mature T cell apoptosis. *J. Exp. Med.*, 183: 2109-2117, 1996.

Lenardo, M.J.: Fas and the art of lymphocyte maintenance. *J. Exp. Med.*, 183: 721-724, 1996.

Regulation of Lymphocyte Development by NF-κB/Rel Proteins

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The inhibitor protein IκBα controls the nuclear import of transcription factors that belong to the NF κB/Rel family (1, 2). This signal-dependent pathway is activated during B cell differentiation and ha been implicated in transcriptional induction of the immunoglobulin kappa (Igκ) locus (3). However recent studies with mice deficient for individual NF-κB/Rel subunits suggest that this pathway is eithe comprised of compensatory components or dispensable for the production of mature Igκ+ B lymphocyte (4-6). To circumvent the potential for functional redundancy, precursor B (pre-B) lymphocyte line immortalized with the Abelson Murine Leukemia Virus (AMuLV) were stably transfected with a expression vector that encodes a trans-dominant form of IκBα, termed IκBΔN. This truncated inhibito lacks N-terminal sequences required for signal-dependent degradation but retains its Rel binding functio (7). In pre-B cells stimulated with bacterial lipopolysaccharide (LPS), IκBΔN constitutively represse the nuclear import of NF-κB/Rel complexes containing the transactivating subunits RelA and c-Rel. Thi dual block in NF-κB/Rel signaling was associated with potent inhibitory effects on germline transcriptio and rearrangement of the endogenous Igκ locus. Similar results were obtained with conditionally transformed pre-B cells expressing a temperature-sensitive mutant of v-abl (8), thus excluding th possibility that these regulatory defects were inextricably linked to the transformed phenotype. Thes findings suggest that c-Rel and RelA play compensatory roles in locus transactivation and recombinatio events that mediate the developmental progression of pre-B cells to mature Igκ-expressing B lymphocyte

Members of the NF-κB/Rel family of transcription factors are also induced during thymic selection and mature T lymphocytes following immune activation (9, 10). To explore the potential role of this signalir pathway in T cell development, we generated transgenic mice that express IκBΔN under lineage-specif control of the proximal lck promoter. Significantly higher steady-state levels of the IκBΔN protein we detected in thymocytes relative to splenocytes from transgenic animals. Biochemical studies indicated th presence of IκBΔN in latent cytoplasmic complexes containing either RelA or c-Rel. Nuclear induction these complexes was constitutively repressed in transgenic thymocytes treated with PMA and ionomycin, combination which mimics activation through the T cell receptor. Consistent with the growth abnormali of c-Rel-deficient T cells (6), this regulatory block led to a dramatic reduction in the proliferative respon of thymocytes to either concanavalin A (con A), combinations of PMA and ionomycin, or immobiliz anti-CD3 antibodies. Addition of exogenous IL-2 failed to restore proliferation to normal levels. Analys of the number and distribution of T cell subsets indicated that disruption of the NF-κB/Rel signali pathway also perturbs the normal balance of CD4 and CD8 single-positive lymphocytes. Specificali numbers of CD8+ T lymphocytes from transgenic mice were disproportionately reduced relative to CD cells in both thymus and spleen. Alteration in the CD4/CD8 ratio was associated with enhanc susceptibility of T lymphoblasts to activation-induced cell death, which compromised CD8+ but not CD cells. Taken together, these asymmetric population dynamics suggest that lineage commitment to matu CD4+ and CD8+ T cell subsets involves distinct NF-κB signaling requirements.

References

1. P. Baeuerle and D. Baltimore. Science 242:540-546, 1988.

2. S. Haskill, A. Beg, S. Tompkins, J. Morris, A. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. Baldwin. Cell 65:1281-1289, 1991.

3. R. Sen and D. Baltimore. Cell 47:921-928, 1986.

4. W. Sha, H. Liou, E. Tuomanen, and D. Baltimore. Cell 80:321-330, 1995.

5. F. Weih, D. Carrasco, S. Durham, D. Barton, C. Rizzo, R. Ryseck, S. Lira, and R. Bravo. Cell 80:331-340, 1995.

6. F. Kontgen, R. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis. Genes Dev. 9:1965-1977, 1995.

7. J. Brockman, D. Scherer, T. McKinsey, S. Hall, X. Qi, W. Lee, and D. Ballard. Mol. Cell. Biol. 15:2809-2818, 1995.

8. Y.-Yi Chen, L. Wang, M. Huang, and N. Rosenberg. Genes Dev. 8:688-697, 1994.

9. J. Sen, L. Venkataraman, Y. Shinkai, J. Pierce, F. Alt, S. Burakoff, and R. Sen. J. Immunol. 154:3213-3221, 1995.

10. N. Moore, J. Girdlestone, G. Anderson, J. Owen, and E. Jenkinson. J. Immunol. 155:4653-4660 1995.

REGULATION AND FUNCTION OF NFκB DURING B CELL DIFFERENTIATION

Thomas Wirth

We have previously demonstrated that primary lymphoid organs contain predominantly constitutively active p50/RelB and p52/RelB complexes, whereas p50/RelA complexes represent the major inducible species (3, 4). I will describe recent experiments, where we investigated activation of NF-κB in the process of primary B cell differentiation. We have employed a primary pre B cell culture/differntiation system developed by Rolink et al. (6). In this system, pre B cells can be expanded in the presence of IL7 and stroma cells, differentiation can be induced by IL7 withdrawal. We show that during *in vitro* differentiation of primary B lymphocytes, NFκB is strongly induced. There are several notable differences between this differentiation-associated induction of NFκB as compared to the "typical" NFκB activation by various exogenous stimuli. This activation proceeds with a slow time courseand specifically involves IκBβ degradation. Activation is only observed in B cells that undergo differentiation and therefore does not occur in Rag2-deficient pre B cells, which cannot rearrange their Ig-loci and therefore are unable to differentiate. The time course of induction of several NFκB target genes closely follows the observed induction of NFκB DNA binding activity. Inducible DNA complexes predominantly comprise p50/RelA heterodimers and, to a much lower extent, c-Rel containing dimers. The increase in NF-κB binding activity is accompanyied by a slow and steady decrease of IκBβ protein levels. Interestingly, absolute RelA protein levels remain unaffected, whereas RelB and c-Rel are induced at the level of protein synthesis. The reason for the preferential induction of nuclear RelA containing complexes appears to be selective inhibition by the IκBβ protein. We demonstrate that IκBβ can efficiently inhibit p50/RelA complexes, but has a much reduced capability to interfere with p50/RelB or p50/c-Rel DNA binding both *in vitro* and *in vivo*. Consistent with this observation, IκBβ cotransfection can inhibit p50/RelA mediated transactivation but barely affects p50/RelB mediated transactivation.

Furthermore, we have investigated the contribution of NFκB to Igκ-locus demethylation. The immunoglobulin κ gene is methylated in most cell types of the mammalian organism and becomes specifically demethylated during B cell maturation (5). Demethylation appears to be a prerequisite for specific gene activation. This reaction requires discrete cis acting elements which include the intronic κ enhancer, as well as the neighboring putative matrix attachment region. We now demonstrate by mutation analysis that the NFκB binding site within the enhancer is essential for initiating cell-type-specific demethylation, indicating that members of the NFκB/Rel family of transcription factors may play a role in this process. In keeping with this hypothesis, the S107 plasmacytoma cell line, which was previously shown to lack nuclear

κB-binding activity (1, 2), also fails to demethylate the κ locus. However, this defect, which has now been characterized at the molecular level, can be complemented by stably expressing RelB in S107 cells, thus fully restoring the demethylation reaction. This represents the first demonstration of a transacting factor involved in confering cell-type-specific demethylation.

References

1. Atchison, M. L., and R. P. Perry, (1987). The role of the κ enhancer and its binding factor NF-κB in the developmental regulation of κ gene transcription. Cell **48:**121-128.

2. Atchison, M. L., and R. P. Perry. (1988). Complementation between two cell lines lacking k enhancer activity: implications for the developmental control of immunoglobulin transcription. EMBO J. **7:**4213-4220.

3. Lernbecher, T., B. Kistler, and T. Wirth. (1994). Two distinct mechanisms contribute to the constitutive activation of RelB in lymphoid cells. EMBO J. **13:**4060-4069.

4. Lernbecher, T., U. Müller, and T. Wirth. (1993). Distinct NF-κB/Rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature **365:**767-770.

5. Lichtenstein, M., G. Keini, H. Cedar, and Y. Bergman. (1994). B cell-specific demethylation: a novel role for the intronic κ chain enhancer sequence. Cell **76:**913-923.

6. Rolink, A., A. Kudo, H. Karasuyama, Y. Kikuchi, and F. Melchers. (1991). Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells *in vitro* and *in vivo*. EMBO J. **10:**327-336.

Session: 6

Chairperson: Ulrich Siebenlist

Signal Transduction in Dorsoventral Patterning of the *Drosophila* Embryo.
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Dorsoventral polarity in the syncytial *Drosophila* embryo is established by a maternally encoded signal transduction pathway (reviewed in Morisato and Anderson, 1995). Activation of the transmembrane receptor Toll in ventral portions of the embryo leads to signal transduction via the novel protein Tube and the protein kinase Pelle. Signaling frees the Rel-related protein Dorsal from its cytoplasmic inhibitor Cactus. Free Dorsal then translocates into ventral and ventrolateral nuclei and directs expression of ventral-specific genes. Our recent work has focused on the role of protein localization, phosphorylation, and degradation in this signaling pathway.

We and others have previously demonstrated that Tube and Pelle interact and that both proteins are constitutively activated fusion to a cell surface receptor (Großhans et al., 1994; Galindo et al, 1995). To determine whether cell surface localization plays a role in wild-type signaling, we have examined the distribution of Tube and Pelle in embryos during establishment of the dorsoventral axis. We find that Tube associates with the cell surface in a graded fashion, with highest concentrations at positions where the signaling pathway is most active. There is also some asymmetry in the distribution of Pelle, although the localization pattern is more complex. On the basis of these results, we hypothesize that activation of Toll by its extracellular ligand directs recruitment of Tube to the cell surface and that binding of Pelle to membrane associated Tube triggers signaling.

Genetic evidence indicates that Pelle-mediated signaling to Dorsal occurs primarily, if not entirely, through Cactus (Roth et al, 1991; Belvin and Anderson, 1995). In the course of immunochemical analyses we have found that signaling induces the spatially graded degradation of Cactus. Using a tissue culture system which reconstitutes Pelle-dependent Cactus degradation, we have demonstrated that a motif in Cactus resembling the sites of signal-dependent phosphorylation in the vertebrate homologs IκB-α and IκB-β (Brown et al, 1995; DiDonato et al., 1996) is essential for Pelle-induced Cactus degradation. Substitution of four serines within this motif with alanine residues generated a mutant Cactus that still functions as a Dorsal inhibitor but is resistant to induced degradation. Injection of RNA encoding this altered form of Cactus has a dominant negative effect on establishment of dorsoventral polarity in the embryo. We conclude that dorsoventral signaling results in a Cactus concentration gradient and propose that signal-dependent phosphorylation directs the spatially regulated proteolysis of Cactus protein.

Cactus is also subject to signal independent degradation (Whalen and Steward, 1993; Belvin and Anderson, 1995). We have found that this proteolysis, which serves to control the level of free Cactus in the embryo, also requires phosphorylation. Using an in-gel assay, we have identified a Cactus kinase activity in embryonic extracts. Deletion mapping indicates that the site of phosphorylation lies in the carboxy-terminal PEST region; phosphoamino acid analysis and site-directed mutagenesis experiments indicate involvement of specific serine residues within the PEST domain. The purified kinase is composed of two subunits of molecular weight 28,000 and 37,000; the larger subunit is responsible for the catalytic activity observed in the in-gel assay. Based on subunit size and composition, site specificity, and amino-terminal peptide sequence, we have identified the Cactus PEST kinase as *Drosophila* CKII (Glover et al, 1983; Saxena et al., 1987). We are currently employing the embryo microinjection assay to investigate the role of CKII-catalyzed modification of the PEST domain in the generation of the Cactus and Dorsal gradients.

References

Belvin, M.P., Jin, Y., and Anderson, K.V. (1995). Cactus protein degradation mediates *Drosophila* dorsal-ventral signaling. Genes Dev. *9*, 783-793.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science *267*, 1485-1488.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996). Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. Mol Cell Biol. *16*, 1295-1304.

Galindo, R.L., Edwards, D.N., Gillespie, S.K.H., and Wasserman, S.A. (1995). Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in *Drosophila* embryos. Development *121*, 2209-2218.

Glover, C.V., Shelton, E.R., and Brutlag, D. L. (1983). Purification and characterization of a type II casein kinase from *Drosophila melanogaster*. J. Biol. Chem. *258*, 3258-3265.

Großhans, J.A., Bergman, A., Haffter, P., and Nüsslein-Volhard, C. (1994). Activation of the kinase Pelle by Tube in the dorsoventral signal transduction pathway of *Drosophila* embryo. Nature *372*, 563-566.

Morisato, D., and Anderson, K.V. (1995). Signaling pathways that establish the dorsal-ventral pattern of the *Drosophila* embryo. Ann. Rev. Genet. *29*, 371-399.

Roth, S., Hiromi, Y., Godt, D., and Nüsslein-Volhard, C. (1991). *cactus*, a maternal gene required for proper formation of the dorsoventral morphogen gradient in *Drosophila* embryos. Development *112*, 371-388.

Saxena, A., Padmanabha, R., and Glover, C.V.C. (1987). Isolation and sequencing of cDNA clones encoding alpha and beta subunits of *Drosophila melanogaster* casein kinase II. Mol. Cell. Biol. *7*, 3409-3417.

Whalen, A.M. and Steward, R. (1993). Dissociation of the dorsal-cactus complex and phosphorylation of the dorsal protein correlate with the nuclear localization of dorsal. J. Cell Biol. *123*, 523-534.

Phosphorylation and the regulation of the Dorsal protein in *Drosophila melanogaster*. Ruth Steward and Eric A. Drier The Waksman Institute, Dept. of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08855 (908) 445-3918.

The embryonic dorsal-ventral axis is established by a maternally encoded signal transduction pathway, that culminates in the graded nuclear localization of Dorsal. In response to a ventral signal, transduced at least in part by the Tube and Pelle proteins, Dorsal dissociates from Cactus and translocates to the nuclei. We have established that the correct ratio of Dorsal and Cactus proteins present in the early embryo is essential for the formation of a normal nuclear Dorsal gradient. This balance appears to be maintained through the degradation of unbound Cactus protein.

We have determined that the RH domain is sufficient to form a normal nuclear gradient, but that N-terminal deletions of the RH domain result in a protein that does not target to the nucleus, even in the absence of *cactus* function. In these deleted proteins, the interaction with Cactus and the homo-dimerization does not appear to be affected. Both Dorsal and Cactus are phosphoproteins and each is multiply phosphorylated We are trying to understand the role that phosphorylation plays in regulating Dorsal function. We have altered several conserved putative phosphorylation sites within the RH domain and established transgenic lines to study their activity. Thus far only one mutation (S312A) has produced a measurable effect on Dorsal activity. The nuclear targeting of this protein is not affected, the mutant protein makes a wild-type gradient, but its function is compromised. We have also altered Dorsal so that it will be either constitutively cytoplasmic or nuclear. We are studying the behavior of these altered proteins in various mutant backgrounds to determine the subcellular location of Dorsal phosphorylation as well as to understand the role of the signal transduction system in regulating this phosphorylation. Our results indicate that Dorsal and Cactus are phosphorylated *in vivo* on serine during oogenesis and early embryogenesis.

Origins of immunity: A novel NF-kB-like gene in *Drosophila* antibacterial defense and embryogenesis

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Although insects lack immunoglobulins, they react to infection by inducing a battery of bactericidal proteins and peptides, including the cecropins. This response may serve as a model system for innate immune reactions in other animals, including man. To find genes that regulate the immune response we screen for differentially expressed transcripts, using the PCR-based differential display technique.

Several new genes have been found, including *Relish*, a compound *Drosophila* protein which, like mammalian p105 and p100, contains both a Rel homology domain and an IkB-like domain. Relish is more than 50-fold induced in infected flies, and it can activate transcription from the *Cecropin A1* promoter. Relish is also detected in early embryos, suggesting that it acts in both immunity and embryogenesis. The presence of a compound Rel protein in *Drosophila* indicates that similar proteins were likely present in primordial immune systems and may serve unique signaling functions.

Regulation of the dorsal silencer in the Drosophila embryo

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The dorsal regulatory gradient initiates the differentiation of the embryonic mesoderm, neuroectoderm, and dorsal ectoderm during early development. Dorsal defines these tissue territories by regulating the expression of various target genes in a concentration-dependent fashion. It activates genes such as twist, snail, and rhomboid, and represses the expression of zen, dpp, and tolloid. Dorsal is an intrinsic activator, but functions as a repressor in the context of certain promoter regions. A distal element in the zen promoter region, located between 1.4 and 1.2 kb upstream of the transcription start site, contains binding sites for both dorsal and closely linked "corepressors". This distal element has the properties of a silencer, and can function over long distances (>5 kb) to repress the ventral expression of various heterologous enhancers, including the eve stripe 2 enhancer. Silencer activity depends on helical phasing of dorsal and neighboring corepressor sites, so that subtle changes in spacing reduce or eliminate repressor function. The long-range silencing exhibited by dorsal-corepressor "complexes" is distinct from other repressors operating in the early Drosophila embryo, including the snail, Kruppel, and knirps repressors. These latter repressors function only over short distances, and must bind within 50-100 bp of upstream activators or the basal promoter to inhibit gene expression. We present evidence that the suHw insulator element in the gypsy retrotransposon fails to block the dorsal silencer, but efficiently impedes the activities of distal (not proximal) enhancers.

POSTERS

ABSOLUTE DEPENDENCE ON κB RESPONSIVE ELEMENTS FOR INITIATION AND TAT-MEDIATED AMPLIFICATION OF HIV TRANSCRIPTION IN BLOOD CD4 T LYMPHOCYTES.

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The role of NF-κB-dependent signals in activating the transcriptional activity of the HIV regulatory region (LTR) was analyzed by systematic comparison of HIV LTR activity in human CD4 T cells purified from peripheral blood and a transformed lymphoblastoid T cell line. In normal CD4 T cells we also analyzed the role played by the viral κB-responsive elements in HIV replication. Analysis of nuclear extracts of resting, normal T lymphocytes revealed the presence of the p50, but not the p65, NF-κB subunit and the induction by phorbol esters of *bona fide* (p50/p65) NF-κB complexes. In parallel, we observed clear enhancer-dependent HIV LTR transactivation comparable in intensity to that observed in lymphoblastoid cells. We show that unstimulated CD4 T lymphocytes offer a cellular environment of very low permissivity to the HIV-LTR functioning. This was in sharp contrast to the high spontaneous LTR activity observed in lymphoblastoid T cells, where LTR activity was essentially independent of κB-responsive elements. Due to the low basal LTR activity in resting T lymphocytes, NF-κB-dependent transactivation was a *sine qua non* event for induction of the HIV-LTR. Surprisingly, even the function of HIV Tat in resting CD4 T lymphocytes was found to be absolutely dependent on LTR κB-responsive elements. The relevance of these observations obtained in transient transfections was confirmed by the incapacity of blood CD4 T lymphocytes infected with an HIV-infectious provirus carrying critical point mutations in the kB-responsive elements to show any detectable transcriptional activity upon cell activation and prolonged culture "in vitro". Our observations emphasize the importance of analyzing the functioning of HIV regulatory domains in the natural environment provided by normal CD4 T lymphocytes to HIV infection, and demonstrate an absolute requirement of NF-κB responsive elements for Tat-dependent and Tat-independent HIV transcription in blood CD4 T lymphocytes.

p100/NF-κB2-MEDIATED INHIBITION OF NF-κB INDUCIBILITY IN BREAST CANCER CELL LINES

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We are investigating the role of the NF-κB family members in the molecular control of the transformed phenotype. Western Immunoblots demonstrated high expression of the p52 precursor p100 (NFKB2) in several breast cancer cell lines. Eighteen primary breast tumors out of 24 displayed significant expression of the p100/p52 protein. In MDA-MB-435 cells, overexpressed p100 and p52 are predominantly cytoplasmic and coimmunoprecipitation experiments demonstrated that p100 sequesters the heterodimer p50/p65 in the cytoplasm. We demonstrated that most p65 protein is complexed with p100 in these cells while it is complexed predominantly with IκB-α in the MCF7 A/Z cells which express less p100. Transient transfections with a NF-κB-dependent reporter plasmid showed that, in cells expressing high levels of p100, transactivation cannot be induced by NF-κB activators such as PMA, TNF-α, IL-1β or H_2O_2, although cytoplasmic IκB-α is degraded following these treatments. In the same cells, we detected significant amounts of IκB-α in nuclear extracts suggesting that free IκB-α can translocate to the nucleus and exert a second level of control on NF-κB activity. We suspect that highly expressed p100 compete with cytoplasmic IκB-α for sequestering NF-κB complexes, thus allowing free IκB-α to translocate to the nucleus. In another hand, the NF-κB-dependent transactivation is increased by NF-κB inducers in the MCF7 A/Z cell line but can be completely abolished by co-transfections of p100. Our data suggest that, when expressed at high levels, the p100/p52 NF-κB subunit could modify the the NF-κB inducibility pathway in human breast cell lines.

DISTINCT SIGNAL TRANSDUCTION PATHWAYS MEDIATE NF-κB INDUCTION BY IL-1β IN EPITHELIAL AND LYMPHOID CELLS

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A large body of work has been devoted to TNF-α or IL-1β signaling leading to the activation of the transcription factor NF-κB. Some studies have indicated that NF-κB induction follows the activation of neutral sphingomyelinase and 5-lipoxygenase and depends strictly on the production of reactive oxygen intermediates (ROIs). However, other authors have demonstrated that TNF-α or IL-1β induce NF-κB through the acidic sphingomyelinase pathway. In this report, we first demonstrated that in the ovarian carcinoma cell line OVCAR-3, IL-1β rapidly induces NF-κB through a complete degradation of IκB-α while H_2O_2 activates NF-κB with a slower kinetics through a partial degradation of IκB-α, p100 and p105. We confirmed that IL-1β-mediated induction of NF-κB in OVCAR-3 and in other epithelial cell lines does not proceed through the production of ROIs but via the acidic sphingomyelinase pathway as it is blocked by inhibition of receptor internalization, as well as by D609, NH_4Cl and chloroquine. However, IL-1β activates NF-κB in lymphoid cells through the intracellular generation of H_2O_2. Our study demonstrated that several signaling pathways lead to the activation of NF-κB following IL-1β treatment in different cell types.

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Apoptosis modulation in AT cells by regulation of NF-κB

The human genetic disease, ataxia telangiectasia (AT), is characterized by neurological degeneration, immunological deficiencies, and exquisite sensitivity to ionizing radiation. Each of these defects may, in part, be the result of inappropriate cellular activation of apoptosis (S. Meyn). In previous studies, we observed constitutive activation of NF-κB in SV40 T-antigen immortalized AT fibroblasts. Furthermore, expression of a transfected truncated IκB-α into the cells resulted in correction of radiation biological defects and regulated NF-κB activation (Science 268; 1619-1621, 1995). We tested these AT cells for induction of apoptosis by exposure to ionizing radiation, and the radiomimetic drug, streptonigrin (STNG). High levels of apoptosis were observed in AT fibroblasts/24 and 48 hours following exposure to ionizing radiation or STNG. However, AT fibroblasts expressing truncated IκB-α show much lower levels of apoptosis, similar to control cells. Analysis of proteins associated with the apoptosis process, including p53, p21, bcl-2, bax, Rb, suggest that this is a p53-independent process. These data support a central role for NF-κB/IκB-α in the apoptotic response of AT cells to ionizing radiation and STNG.

INHIBITION OF NF-KB ACTIVATION BY A VIRAL ENCODED I-KB-LIKE PROTEIN OF AFRICAN SWINE FEVER VIRUS. A NEW MECHANISM OF EVASION OF INNATE IMMUNITY

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Cytokines play a central role in the innate immunity mostly through induction of NF-KB. On the other hand, there are many examples of viruses that have evolved mechanism to counteract the actions of cytokines. The African Swine Fever virus (ASFV), a large DNA deoxyvirus encodes a protein (A238L) of 28.2 KDa which contains ankyrin repeats similar to those of IK-B proteins inhibitors of NF-KB. Here we present evidence that A238L (I-KB ASFV) gene transfected into cells inhibits TNF-induced activation of a reporter gene under control of KB-sites. Moreover, purified recombinant A238L protein prevented binding of p65/p50 NF-KB to their target sequence in the DNA. Furthermore, ASFV-infected cells have an impaired ability to activate NF-KB induced by TNF. A238L was associated to p65/p50 NF-KB in those infected cells. These results strongly suggest the ASFV have A238L gene to interfere with NF-KB activation representing a new mechanism to evade immune response during viral infection.

BIPHASIC CONTROL OF NUCLEAR FACTOR-kB ACTIVATION DURING T CELL ACTIVATION

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The regulation of nuclear factor (NF)-kB activation by the T cell receptor/CD3 complex (TCR/CD3) in primary human T cells has been studied at various times after activation. Only p50 NF-kB bound the kB element of interleukin-2 receptor a chain promoter on resting T cells. However, at immediate times (1 h.) after activation, the binding of p50.p65 heterodimers was observed. p50.c-rel heterodimers were also detected bound to this sequence at early times (7-16 h.), and both remained active up to late times (40 h.). This regulation takes place mainly at the level of nuclear translocation of p65 and c-rel, at immediate and early times. Activation also induced c-rel and p105/p50 mRNA synthesis, but not p65 mRNA whose expression was constitutive. All those early and late events, but not the immediate ones, were inhibited by a neutralizing anti-tumor necrosis factor (TNF) antibody. Similarly, Cycloheximide prevented the p65 and c-rel translocation and consequent formation of active binding heterodimers, at early and late times. Cyclosporin A impaired not only early and late, but also immediate events, but addition of TNF prevented all the inhibitions. These results indicate that the regulation of NF-kB activation during T cell activation by TCR/CD3 signals is biphasic: TCR/CD3 triggers its immediate translocation, which is transient if no TNF is present. Then, TNF emerges as the main factor responsible for a second phase of NF-kB regulation, controlling both translocation of p65 and c-rel, and new mRNA synthesis for c-rel and p105/p50.

Studies about the evolution of the IL-1R/NFκB/IκB related Toll/dorsal/cactus signal transduction pathway in insects

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In the fruitfly *Drosophila melanogaster* cell fate along the dorso-ventral axis is controlled by a single maternal signal transduction pathway. Interactions of the ooyte with its surrounding follicle cells give rise to the activation of the membrane receptor Toll at the ventral side of the developing egg. This activation leads to the phosphorylation of Cactus, a protein that inhibits the function of the transcription factor Dorsal by the formation of Cactus/Dorsal complexes in the cytoplasm. After phosphorylation, these complexes dissociate and Dorsal, a transcription factor of the REL-family, translocates to the nucleus where it activates genes like *twist* and represses genes like *dpp*.
This dorso-ventral signal transduction pathway seems to be homologous to the IL-1R/NFκB/IκB signal transduction pathway in the mammalian immune system: there is a high degree of similarity between Dorsal and vertebrate NFκb, between Toll and the cytoplasmic domain of the IL-1 receptor and between Cactus and IκB. In addition, activation is mediated in both cases by similar phosphorylation events, and Dorsal and NFκB bind to the same κB sites in the regulatory regions of their target genes.
Tube, a protein of unknown function and Pelle, a protein kinase, are known as additional players in the dorso-ventral cascade in *Drosophila*. Previous efforts to unambiguously identify the protein kinase activated by the IL-1 receptor and the search for other players in the mammalian pathway, like a *tube* related gene, have been unsuccessful.

To understand more about the evolution of these signal transduction pathways and to help characterise the involved proteins, we decided to study the evolution of the dorso-ventral signal transduction pathway in closely related animals, namely, various insect species.
Using a PCR approach we obtained sequences for *Toll* and *dorsal* from the midge *Clogmia albipunctata* (Psychodidae, Diptera) and from *Thermobia domestica* (Lepismatidae, Thysanura), a silverfish-like apterygote insect.
cDNA sequence and mRNA expression pattern of the *Toll* homologue in *Clogmia albipunctata* show a high degree of homology to *Drosophila Toll* and reveal some highly conserved and, therefore, putative important protein domains. A sequence analysis of *dorsal* related cDNA clones and expression studies with a cross reacting REL-domain antibody are currently in progress as are functional experiments in *Drosophila* (rescue of *Toll* or *dorsal* mutants) and *Clogmia* (dominant gain of function mutations) to reveal information about the degree of functional conservation of the factors and conserved protein domains. Similar studies with *tube* and *pelle* and with *twist* and *dpp* are planned.

NUCLEAR LOCATION OF IκB-α IN T LYMPHOCYTES

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The Rel/NF-κB family of transcription factors plays an esential role in the regulation of the HIV replication in T lymphocytes. NF-κB is composed of heterodimeric and homodimeric complexes of Rel proteins p50, p52, p65, Rel B and c-Rel. Typically, NF-κB is localized in the cytoplasm complexed with members of the IκB family.

The induction of Rel/NF-κB activity by different stimuli (antigen recognition, cytokines as TNF and IL-1 or PMA), involves the release of IκB from the Rel/NF-κB complexes, allowing their rapid translocation to the nucleus where they can transcriptionally regulate target genes.

In this work, we have analysed the location of IκB-α (MAD-3) in peripheral blood T lymphocytes, both in resting and PMA stimulated cells.

Human mononuclear cells were isolated from whole blood of healthy donors by centrifugation in Ficoll-Hypaque. T cells were purified by passing through to nylon fiber column and purified T cells were activated with PMA (25 ng/ml) at different times.

Nuclear and cytoplasmic extracts were analysed by Western-Blot. Cytosolic contamination of nuclear proteins was excluded using an anti-LDH antibody. Moreover, slides of T cells were fixed, labelled with specific antibodies and visualized by confocal microscopy.

Resting T lymphocytes show a predominant cytosolic location of IκB-α and NF-κB proteins. However, in the nuclei of T lymphocytes, we detect the presence of p50 homodimers, p65 and small concentrations of IκB-α. PMA activation induced a permanent translocation of NF-κB in the nuclei of T cells. The results of Western-Blot and confocal microscopy demonstrate also an increase in the nuclear location of IκB-α in activated T cells.

In summary, our results show that in T cells, IκB-α is present in the nucleus of both, resting and activated T lymphocytes.

These results suggest a new role of IκB-α in NF-κB regulation. Nuclear IκB-α could be involved in the transport of NF-κB or act as a nuclear regulatory protein of the Rel/ NF-κB family.

Regulation of NF-κB Activity via IκBα Phosphorylation and Degradation

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The NF-κB/Rel transcription factors participate in the activation of immune regulatory genes and viral early genes including the HIV-1 LTR. Cell activation leads to the phosphorylation and degradation of IκBα, permitting NF-κB/Rel translocation to the nucleus and target gene activation. To further characterize the signaling events that contribute to IκBα phosphorylation, a kinase activity was isolated from Jurkat T cells that specifically interacted with IκBα in an affinity chromatography step and phosphorylated IκBα with high specificity *in vitro*. Using an in gel kinase assay with recombinant IκBα as substrate, two molecular weight forms of the kinase (43 and 38kDa) were identified. Biochemical criteria and immunological cross-reactivity identified the kinase activity as the alpha catalytic subunit of casein kinase II. Deletion mutants of IκBα localized phosphorylation to the C-terminal PEST domain of IκBα. Point mutation of residues T291, S283 and T299 dramatically reduced phosphorylation of IκBα by the kinase *in vitro*. Constitutive phosphorylation of the triple point mutant was eliminated *in vivo*, although TNF inducible IκBα degradation was unaffected. In cell lines and in transiently transfected cells, mutation of the CKII sites in IκBα resulted in a protein with increased intrinsic stability. IκBα deletion studies in NIH 3T3 cells demonstrated that the C-terminal 30 amino acids (aa288 to aa317, deleted in IκBαΔ3), including most of the PEST domain, were dispensible for IκBα function. However, deletions from aa261 to aa317 or aa269 to aa317 (IκBαΔ1 and IκBαΔ2), lacked the ability to dissociate NF-κB/DNA complexes *in vitro* and were unable to inhibit NF-κB dependent transcription. Moreover, IκBαΔ1 and IκBαΔ2 were resistant to inducer mediated degradation. Analysis of IκBα deletions in the presence of protein synthesis inhibitors revealed that, IκBαΔ1 and IκBαΔ2 had a half life four times shorter than wild type IκBα and the interaction with p65 was dramatically decreased *in vivo* as measured by co-immunoprecipitation. Interestingly, protease inhibitors which block inducer mediated degradation of IκBα also stabilized the turnover of IκBαΔ1 and IκBαΔ2. Based on these studies, we propose that in the absence of stimulation, the C-terminal domain (aa269 to aa287) may play a role to protect IκBα from a constitutive protease activity. We also examined the effect of transdominant mutants of IκBα on the synergistic activation of the HIV-1 LTR by TNFα and the HIV transactivator, Tat, in Jurkat T cells. The synergistic induction of HIV LTR driven gene expression represented a 50-70 fold stimulation and required both an intact HIV-1 enhancer and Tat-TAR element interaction. Co-expression of IκBα inhibited Tat-TNF activation of HIV LTR in a dose dependent manner. Transdominant forms of IκBα, mutated in critical serine or threonine residues required for inducer mediated (S32A,S36A) and/or constitutive phosphorylation (S283A,T291A,T299A) of IκBα were tested for their capacity to block HIV LTR transactivation. IκBα molecules mutated in the N-terminal sites were not degraded following inducer mediated stimulation (T1/2 >4 hours) and were able to efficiently block HIV LTR transactivation. Strikingly, the IκBα(S32A,S36A) transdominant was at least 5 times more effective than wtIκBα in inhibiting synergistic induction of the HIV-LTR. This mutant also effectively inhibited HIV-1 multiplication in a single cycle infection model in Cos-1 cells, as measured by Northern blot analysis of viral mRNA species and viral protein production. These experiments suggest a strategy that may contribute to inhibition of HIV-1 gene expression by interfering with the NF-κB/Rel signaling pathway.

TRANSCRIPTIONAL REGULATION OF THE GENE ENCODING THE HUMAN LEUKOCYTE ACTIVATION ANTIGEN CD69. FUNCTIONAL CHARACTERIZATION OF ITS TNF-α RESPONSIVE ELEMENTS.

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The human activation antigen CD69 is a member of the C-type animal lectin superfamily that functions as a signal-transmitting receptor. Although the expression of CD69 can be induced *in vitro* on cells of most hematopoietic lineages with a wide variety of stimuli, *in vivo* it is mainly expressed by T lymphocytes located in the inflammatory infiltrates of several human diseases. To elucidate the mechanisms that regulate the constitutive and inducible expression of CD69 by leukocytes, we isolated the promoter region of the CD69 gene, and carried out its funtional characterization. Sequence analysis of the 5'-flanking region of the CD69 gene revealed the presence of a potential TATA element 30bp upstream of the major transcription initiation site and several putative binding sequences for inducible transcription factors (NF-kB, AP-1, Egr-1), which might mediate the inducible expression of this gene. Transient expression of CD69 promoter-based reporter gene constructs in K562 cell line, indicated that the proximal promoter region spanning from -78 to +16 contained the *cis*-acting sequences necessary for basal and PMA-inducible transcription of CD69 gene. The removal of the upstream sequences located between -78 and -38, resulted in a decreased promoter strength and abolished the response to PMA. We also found that TNF-α is capable to induce the surface expression of CD69 molecule, as well as the promoter activity of fusion plasmids that contain 5'-flanking sequences of the CD69 gene, suggesting that this cytokine may regulate *in vivo* the expression of CD69 antigen. In addition, cotransfection experiments demonstrated that the CD69 gene promoter can be activated the NF-kB family members c-rel and relA. The deletion of the sequence spanning from -255 to -170 abolished both the response to TNF-α and the trans-activation by NF-kB. These results indicate that the NF-kB binding site located at position -223 is necessary for the TNF-α-induced expressión of the CD69 gene. Mobility shift assays showed the two NF-kB motifs located in the proximal promoter region (-223 and -160) bind variuos NF-kB-related complexes including the heterodimers p50/relA and p50/c-Rel, and homodimers of P50 (KBF1) and relA. Our findings help to understand the regulated synthesis of CD69 *in vivo* and suggest that TNF-α has a key role in the expression of this molecule a the sites of chronic inflammation.

IN VIVO REGULATION OF REL PROTEIN-DNA INTERACTION BY NUCLEAR IκBα

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Rel family proteins have been implicated in normal lymphoid cell function as well as in their transformation. These proteins bind to κB DNA sites, form heterodimers with one another, and modulate the expression of genes linked to κB DNA motifs. THe IκB family of Rel protein inhibitors is important for the regulation of these activities. IκB factors have been shown to inhibit Rel DNA-binding *in vitro*, to displace DNA from DNA-bound Rel complexes and to inhibit *in vitro* transcription reactions. Previous studies from this group showed that IκBα functionally interacts with the conserved RxxRxRxxC Rel DNA-binding motif to inhibit Rel association with DNA *in vitro*. These findings raised the possibility that nuclear IκB factors may also regulate the transcriptional activity of Rel proteins *in vivo*. According to this model, IκBα may act as an ON/OFF switch to control Rel protein activity in the cytoplasm and in the nucleus. Given the significant implications of this model, it is important to investigate the regulation of Rel proteins by nuclear IκB factors and to examine their biological activity.

Our approach to address this issue has been to bypass the effects of IκB on the cytoplasmic regulation of Rel. To this end, we have designed Rel proteins that constitutively localize to the nucleus and compared the DNA binding, transcriptional and biological activities of these proteins to those of nuclear Rel mutants defective for association with IκBα.. As predicted by our model, transfection assays of wt or mutant Rel genes alone or together with IκBα demonstrated that IκBα can directly regulate the transcriptional activity of Rel proteins in the nucleus. Ongoing and future experiments will help to further clarify the role that the nuclear regulation of Rel by IκBα plays in the biological activities of the v-Rel and c-Rel proteins.

Evidence for the involvement of NF-κB activation in the inducible expression of selected genes in human neutrophils.

by Patrick P. McDonald and Marco A. Cassatella

Neutrophils rank amongst the first blood cells that migrate towards inflammatory lesions, where they accumulate in large numbers and perform host defence functions. Activated neutrophils have the ability to upregulate the expression of many genes, in particular those encoding cytokines and chemokines, and to subsequently release the corresponding proteins. Although little is known to date concerning the regulation of gene transcription in neutrophils, it is noteworthy that many of these genes depend upon the activation of transcription factors, such as NF-κB, for inducible expression. We therefore investigated whether NF-κB/Rel proteins are expressed in human neutrophils, as well as their fate upon cell activation. We now report that p50/NFκB1, p65/RelA, and c-Rel are present in neutrophils, and that the greater part of these proteins is physically associated with cytoplasmic IκB-α in resting cells. Whereas no change in the cellular levels of NF-κB/Rel proteins could be detected in cycloheximide-treated neutrophils (for up to 2.5 h), the IκB-α protein was found to turn over relatively rapidly, as its estimated half-life was of approximately 75 min. Accordingly, resting neutrophils expressed elevated constitutive levels of mRNA encoding IκB-α. Following the stimulation of neutrophils with pro-inflammatory agonists that induce the production of cytokines and chemokines in these cells, NF-κB/Rel proteins translocated to nuclear fractions, resulting in enhanced nuclear NF-κB DNA-binding activities. This redistribution was closely paralleled by IκB-α phosphorylation and subsequent proteolysis. Under these conditions, activated neutrophils also accumulated mRNA transcripts encoding IκB-α, which resulted in the re-expression of the IκB-α protein, in a protein synthesis-dependent manner. Finally, pretreatment of neutrophils with AEBSF prevented the inducible IκB-α proteolysis, nuclear accumulation of NF-κB/Rel proteins, and NF-κB activation, as well as the accumulation of mRNA transcripts from κB-dependent genes such as the ones encoding IκB-α and IL-8. To our knowledge, this constitutes the first evidence that NF-κB activation may underlie the action of pro-inflammatory stimuli towards human neutrophil gene expression.

Mechanisms of Ubiquitin-Proteasome Mediated Procesing of the NF-κB1 Precurssor, p105

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Several transcription factors, NF-κB among them, are activated or inactivated by specific proteolytic events. We set to study the role of the ubiquitin system in the activation of NF-κB. Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete ATP-dependent steps: **(a)** Covalent attachment of multiple ubiquitin molecules to the protein substrate, and **(b)** Degradation of the targeted protein by a 26S proteasome complex

In most cases, the transcriptional factor NF-κB is a heterodimer that is composed of two subunits, p50 and p65, which are encoded by two distinct genes of the *rel* family. p50 is translated as a precursor of 105 kDa. The C-terminal domain of the precursor is rapidly degraded, forming the mature p50 subunit consisted of the N-terminal region of the molecule. The mechanism of generation of p50 is not known. It has been suggested that the ubiquitin-proteasome system is involved in the process.

Recently, we were able to reconstitute a cell free processing system and show directly that: (a) the ubiquitin-proteasome system is involved in processing of the intact p105 precursor; (b) conjugation of ubiquitin to the precursor is an essential intermediate step in the processing; (c) a novel, ~320 kDa species of ubiquitin-protein ligase that was purified from rabbit retyclocyte lysate is involved in the process. This novel enzyme is required along with a ubiquitin-carrier protein from the ubc5/E2-F1 family for tagging the p105 to processing by the 26S proteasome. The ligase is distinct from E6-AP, the p53 conjugating ligase, from E3α, the "N-end rule" ligase, and from a novel E3 involved in the degradation of the transcriptional activator MyoD; (d) A unique sequence within the p50 molecule serves, most probably, as a "processing signal" that directs limited proteolysis rather the complete degradation of the molecule.

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Regulation of IkBα in HIV-Infected Macrophages. Role of the IkBα Kinase PKCK2

Macrophages contrary to T cells serve as the major reservoir of persistent HIV replication. Persistent activation of NF-kB in HIV-infected macrophages is a main mechanism whereby viral persistence is regulated. Previous work from our group has identified that IkBα and recently IkBβ are selectively targeted by HIV infection by HIV. The half life of these human IkBs is significantly reduced in infected cells, which results in continuous NF-kB translocation. IkBα is found to be hyperphosphorylated in its C-terminus through an S32/36 independent mechanism. Using a variety of biochemical approaches, we have characterized the phosphorylation sites in the C-terminus as S-283, S-289, S-293, and T-291. Additional studies have identified protein kinase PKCK2 as the one responsible for the C-terminus phosphorylation. Binding studies have also determined that PKCK2 physically interacts *in vivo* and *in vitro* with IkBα through the C-terminal region independently from p65. The functional role of PKCK2 was established in *in vitro* degradation assays indicating that this kinase is necessary for the degradation of IkBα in HIV-infected cells. Current studies are underway to determine whether PKCK2 is required for phosphorylation at the "inducible" S32/36 sites within the N-terminus.

Recent studies from our group have identified second signalling messengers which lie upstream of PKCK2 and that regulate IkBα in HIV-infected monocytes. These include $p21^{ras}$ and Raf-1 kinase as well as the atypical PKC isoform, PKC-ζ/ι. In addition, inhibition of PKC-ζ results in decreased replication in HIV-infected macrophages. Studies are underway to determine direct interactions between PKCK2 and these second signalling messengers.

In summary, our studies indicate that persistent HIV-infected macrophages is a main mechanism regulating viral persistence in these cells, and that the selective upregulation of second messengers lead to the activation of IkBα kinases such as PKCK2 that ultimately impact the phosphorylation and degradation of IkB molecules.

Coupling of p50/C-Rel to the IgH 3´enhancer activity in late B cell development **Sven Pettersson, Ylva Lindersen, Tove Andersson, Marcus Neurath (1) and Daniel Cross. Karolinska Institute, Center for Biotechnology, 141 57 Huddinge, Sweden. (1) Dept of Mol. immunology, Univ. of Mainz, Germany.** The function of the IgH 3´enhancer is likely to be involved in class switch recombination and regulation of IgH gene expression. Here we examine the molecular basis as to how this element execute its biological activities in B cells. Signalling via the IgM- or the CD40-receptor or LPS stimulation can activate the 3´enhancer. *In vivo* footprinting experiments reveals that whilst some motifs becomes occupied irrespective of stimulation, others are protected depending on the stimuly used. Whereas IgM or CD40 receptor stimulation induce the NFAB (Nuclear Factors of Activated B cells) proteins to bind to an Ets/AP-1 site important for enhancer function, LPS and CD40 stimulation induce occupancy of a NF-kB site in the enhancer. Mutagenesis of the NF-kB site revealed that the NF-kB site is important for enhancer activity. We have identified the subcomponents of NF-kB, p50 and C-Rel, to bind to this site in extracts from primary B cells. A minimal promoter containing one copy of the NF-kB-site, linked to a reporter gene, exhibit low levels of transactivating ability in plasma cell lines, suggesting that the regulatory activity of NF-kB is mediated via "crosstalk" with other proteins. Interestingly, NF-kB proteins has been shown to physically interact with Ets proteins. By introduction of ets-like sites from the enhancer into the minimal NF-kB promoter construct, which by genomic foot printing experiments are occupied upon stimulation and by sitedirected mutagenesis display imortatn activity ofr enhancer function, we have identified other factors which act in synergy with Nf-kB in functional assays. Thus, these proteins, together with NF-kB, appears to form strong transcriptional activators and depending on stage in B cell differentiation or receptor stimulation used, NF-kB may interact wtih different proteins. Taken together, we provide direct evidence for the importance of p50/C-Rel in IgH gene expression via the 3´enhancer. Moreover, we have identified a set of proteins which together with NF-kB can potentiate enhancer function that may depend on which ligand-receptor stimulation that is used to trigger enhancer function. We will discuss the data in context of a transcription factor network regulating late B lymphocyte development.

Mechanisms of Proteolytical Degradation of IκBα Regulating NF-κB activity.

Manuel Salvador RODRIGUEZ MEDINA.

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In unstimulated cells, the transcription factor NF-κB is sequestered in the cytoplasm as an inactive complex containing the inhibitor protein IκBα. Activation of NF-κB involves signal-induced degradation of IκBα and release of NF-κB, which translocates to the nucleus where it influences transcription of responsive genes. N and C-terminal regions of IκBα are involved in this process. We found that the acidic C-terminal region controls inducible degradation of the molecule by an unknown mechanism which probably involves structure modifications and phosphorylation. The N-terminal region of the protein has been identified as a regulatory region that is required for signal-induced phosphorylation and degradation. The sensitivity of IκBα degradation to peptide aldehydes which inhibit components of the proteasome and the detection of ubiquitinated forms of IκBα indicate that IκBα is degraded by the ubiquitin proteasome pathway. Following cell signaling IκBα is post-transductionally modified by phosphorylation and conjugation to ubiquitin multichains. Our *In vivo* experiments shown that ubiquitinated forms of the protein dissociate from NF-κB. IκBα mutant carrying serine to alanine changes at amino-acid 32 and 36, blocks both signal-induced phosphorylation and ubiquitin conjugation of the protein. A mutant form of IκBα containing lysine to arginine changes at positions 21 and 22 (K21R, K22R) severely reduces TNF-induced activation of the NF-κB-dependent reporter gene. Combined mutation of two other lysines, K38 and K47, located in the N-terminus of IκBα promoted only a modest increase of its NF-κB-inhibitory capacity. Changing other lysines residues did not reduce activation of the NF-κB-dependent reporter gene by IκBα as compared to the wild type form of the protein. Examination of the metabolism of mutant IκBα molecules reveals that while the K21R, K22R mutant inhibits the DNA binding activity of NF-κB and undergoes signal-induced phosphorylation, it is neither ubiquitinated nor degraded in response to TNF. Thus, it is likely that after signal-induced phosphorylation of IκBα on serine residues 32 and 36, lysine residues 21 and 22 are major sites of ubiquitin ligation which target the protein for rapid degradation by the proteasome.

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Negative cross-talk between the RelA subunit of NFκB and steroid receptors.

Interactions between steroid receptors and other transcription factors are an important means of regulating gene transcription. Previously, we described a negative cross-talk between NFκB and the glucocorticoid receptor (GR). Whereas NFκB proteins function as positive regulators of genes which play a role in cell proliferation and immune response, glucocorticoids down-regulate the expression of these genes.
Since progestins also play a role in these processes, we have investigated possible transcriptional interactive effects of NFκB family members and the progesterone receptor (PR). We observed mutual repression of the RelA subunit of NFκB (p65) and PR. This *trans*-repression is shown to occur independently of PR isoform, reporter construct or cell-type used. With respect to the domains of PR that are involved in repression of RelA, we found that both the DNA-binding domain as well as the ligand-binding domain are essential for repression. Together with the demonstration of interaction between RelA and PR *in vitro*, these findings suggest that the mutual repression is due to a direct interaction between these proteins. Currently, we are investigating which domain of RelA is involved in the repression of both GR and PR.
Since NFκB-regulated cytokine receptors are expressed in progesterone target tissues (breast and endometrium), the mutual repression of PR and RelA could play a role in a wide variety of physiological processes in these tissues, including maintenance of pregnancy, immunosuppression and tumorigenesis.

Stimulation-dependent IkBa phosphorylation marks the NF-kB inhibitor for degradation via the ubiquitin-proteasome pathway

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Nuclear translocation is apparently the major step in the activation of NF-kB which, once it enters the nucleus, is capable of binding its cognate site, thereby initiating the transcription process.A critical step mobilizing the factor into the nucleus is its release from a cytoplasmic inhibitor.-IkB. Phosphorylation of IkB in cell-free experiments results in its inactivation and release from the Rel complex, but *in vivo* NF-kB activation is associated with IkB degradation. The *in vivo* role of phosphorylation is unknown. Our study shows that T-cell activation results in rapid phosphorylation of IkBα and that this event is a physiological one, dependent on appropriate lymphocyte costimulation. Inducible IkBα phosphorylation was abolished by several distinct NF-kB blocking reagents, suggesting it plays an essential role in the activation process. However, the *in vivo* induction of IkBα phosphorylation did not cause the inhibitory subunit to dissociate from the Rel complex. Using an in vitro proteolysis system ,we show that of the two IkBa forms recovered from stimulated cells in complex with RelA, only the newly phosphorylated form, plkBa, is a substrate for an *in vitro* reconstituted ubiquitin-proteasome system. Proteolysis requires ATP, ubiquitin, a specific ubiquitin conjugating enzyme, and other proteasome-ubiquitin components. *In vivo*, inducible IkBa degradation requires a functional ubiquitin activating enzyme, and is associated with the appearance of high molecular weight adducts of IkBa. This is probably a first example of targeting a regulatory protein by the ubiquitin-proteasome system as a signal transduction event.

List of Invited Speakers

Workshop on

NF-κB/IκB PROTEINS. THEIR ROLE IN CELL GROWTH, DIFFERENTIATION AND DEVELOPMENT

List of Invited Speakers

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