Chapter – I

Introduction
Introduction

Proteins are synthesized as large precursors, p105, and p100, which undergo processing to generate the mature NF-κB subunits, p50 and p52, respectively. The processing of p105 and p100 is mediated by the ubiquitin/proteasome pathway and involves selective degradation of their C-terminal region containing ankyrin repeats.

![Diagram of NF-κB protein structure]

**Fig 1: Schematic diagram of NF-κB protein structure.**

Both Class I (top) and class II (bottom) of proteins contain a N-terminal DNA-binding domain (DBD), which also serves as a dimerization interface to other NF-κB transcription factors and, in addition, binds to the inhibitory IκBα protein. The C-terminus of class I proteins contains a number of ankyrin repeats and has transrepression activity. In contrast, the C-terminus of class II proteins has a transactivation function.

NF-κB family members share structural homology with the retroviral oncoprotein v-Rel, resulting in their classification as NF-κB/Rel proteins. There are five proteins in the mammalian NF-κB family.

NF-κB and Proteomics
### Introduction

<table>
<thead>
<tr>
<th>Class</th>
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<th>Aliases</th>
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<tbody>
<tr>
<td>I</td>
<td>NF-κB1</td>
<td>p105 → p50</td>
<td>NFKB1</td>
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<tr>
<td></td>
<td>NF-κB2</td>
<td>p100 → p52</td>
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<td>RelA</td>
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<td>c-Rel</td>
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Table 1: Showing different NF-κB proteins present in mammals.

**Species distribution and evolution:**

In addition to mammals, NF-κB is found in a number of simple animals as well. These include cnidarians (such as sea anemones, coral and hydra), porifera (sponges), the single-celled eukaryote *Capsaspora owczarzaki* and insects (such as moths, mosquitoes and fruitflies). The sequencing of the genomes of the mosquitoes *A. aegypti* and *A. gambiae*, and the fruitfly *D. melanogaster* has allowed comparative genetic and evolutionary studies on NF-κB. In those insect species, activation of NF-κB is triggered by the Toll pathway (which evolved independently in insects and mammals) and by the Imd (immune deficiency) pathway.
**Introduction**

**Signaling:**

NF-κB heterodimerizes with RelB to form a ternary complex with DNA that promotes gene transcription. NF-κB is important in regulating cellular responses because it belongs to the category of "rapid-acting" primary transcription factors, i.e., transcription factors that are present in cells in an inactive state and do not require new protein synthesis to be activated (other members of this family include transcription factors such as c-Jun, STATs, and nuclear hormone receptors). This allows NF-κB to be a first responder to harmful cellular stimuli. Known inducers of NF-κB activity are highly variable and include reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β), bacterial lipopolysaccharides (LPS), isoproterenol, cocaine and ionizing radiation.

![Figure 2: Mechanism of NF-κB action.](image-url)
Introduction

The NF-κB heterodimer present between Rel and p50 proteins is used as an example. While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA, which, in turn, is translated into protein, which results in a change of cell function.

In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm due to the interaction with proteins of the IκB family. Stimulation of cells, e.g. by proinflammatory agents results in the rapid activation of the IκB kinase (IKK) complex. This complex consists of two kinases IKK1/a and IKK2/b as well as a regulatory component called NEMO/IKK-g (Ghosh and Karin, 2002). Its activation results in the de novo phosphorylation of conserved serine residues in the aminoterminal domain of the IκB proteins marking them for ubiquitination and subsequent degradation by the proteasome. This allows nuclear translocation of NF-κB and binding to cognate DNA motifs in the promoter region of target genes, which subsequently initiates transcription of these genes and finally starts a genetic
Introduction

program responsible for e.g. inflammatory responses (Denk et al., 2000). Numerous efforts have been initiated to develop or to identify specific low molecular weight compounds to inhibit this pathway (Garg and Aggarwal, 2002). Substances that inhibit the proteasome as well as radical scavengers have been shown to block NF-κB activation. These inhibitors have been valuable for many studies of NF-κB functions in cell culture systems. However, as these compounds also affect multiple other cellular reactions, they are not useful as therapeutic agents in vivo (Epinat and Gilmore, 1999). Substances that either block the action of the IκB kinase complex or interfere with its activation are thought be more specific and should have fewer side effects.

Receptor activator of nuclear factor kappa B (RANK), which is a type of TNFR, is a central activator of NF-κB. Osteoprotegerin (OPG), which is a decoy receptor homolog for RANK ligand, inhibits RANK by binding to RANKL, and, thus, osteoprotegerin is tightly involved in regulating NF-κB activation. Many bacterial products and stimulation of a wide variety of cell-surface receptors lead to NF-κB activation and fairly rapid changes in gene expression. The identification of Toll-like receptors (TLRs) as specific pattern recognition molecules and the finding that stimulation of TLRs leads to activation of NF-κB improved our understanding of how different pathogens activate NF-κB. For example, studies have identified TLR4 as the receptor for the LPS component of Gram-Negative bacteria. TLRs are key regulators of both innate and adaptive immune responses.
Unlike RelA, RelB, and c-Rel, the p50 and p52 NF-κB subunits do not contain transactivation domains in their C terminal halves. Nevertheless, the p50 and p52 NF-κB members play critical roles in modulating the specificity of NF-κB function. Although homodimers of p50 and p52 are, in general, repressors of κB site transcription; both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel. In addition, p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators.

In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB), which are proteins that contain multiple copies of a sequence called ankyrin repeats. By virtue of their ankyrin repeat domains, the IκB proteins mask the nuclear localization signals (NLS) of NF-κB proteins and keep them sequestered in an inactive state in the cytoplasm. IκBs are a family of related proteins that have an N-terminal regulatory domain, followed by six or more ankyrin repeats and a PEST domain near their C terminus. Although the IκB family consists of IκBα, IκBβ, IκBε, and Bcl-3, the best-studied and major IκB protein is IκBα. Due to the presence of ankyrin repeats in their C-terminal halves, p105 and p100 also function as IκB proteins. The c-terminal half of p100, that is often referred to as IκBδ, also functions as an inhibitor. IκBδ degradation in response to developmental stimuli, such as those transduced through LTβR, potentiate NF-κB dimer activation in a NIK dependent non-canonical pathway.
Introduction

Activation of the NF-κB is initiated by the signal-induced degradation of IκB proteins. This occurs primarily via activation of a kinase called the IκB kinase (IKK). IKK is composed of a heterodimer of the catalytic IKK alpha and IKK beta subunits and a "master" regulatory protein termed NEMO (NF-κB essential modulator) or IKK gamma. When activated by signals, usually coming from the outside of the cell, the IκB kinase phosphorylates two serine residues located in an IκB regulatory domain. When phosphorylated on these serines (e.g. serines 32 and 36 in human IκBα), the IκB inhibitor molecules are modified by a process called ubiquitination, which then leads them to be degraded by a cell structure called the proteasome.

With the degradation of IκB, the NF-κB complex is then freed to enter the nucleus where it can 'turn on' the expression of specific genes that have DNA-binding sites for NF-κB nearby. The activation of these genes by NF-κB then leads to the given physiological response, for example, an inflammatory or immune response, a cell survival response, or cellular proliferation. NF-κB turns on expression of its own repressor, IκBα. The newly synthesized IκBα then re-inhibits NF-κB and, thus, forms an auto feedback loop, which results in oscillating levels of NF-κB activity. In addition, several viruses, including the AIDS virus HIV, have binding sites for NF-κB that controls the expression of viral genes, which in turn contribute to viral replication or viral pathogenicity. In the case of HIV-1, activation of NF-κB may, at least in part, be involved in activation of the virus from a latent, inactive state. YopP
Introduction

is a factor secreted by Yersinia pestis, the causative agent of plague that prevents the ubiquitination of IκB. This causes this pathogen to effectively inhibit the NF-κB pathway and thus block the immune response of a human infected with Yersinia.

Inhibitors of NF-kB activity:

Concerning known protein inhibitors of NF-kB activity, one of them is IFRD1, which represses the activity of NF-kB p65 by enhancing the HDAC-mediated deacetylation of the p65 subunit at lysine 310, by favoring the recruitment of HDAC3 to p65. In fact IFRD1 forms trimolecular complexes with p65 and HDAC3.

Non-canonical-

A select set of cell-differentiating or developmental stimuli, such as lymphotoxin-α, BAFF or RANKL, activate the non-canonical NF-κB pathway to induce NF-κB/RelB: p52 dimer in the nucleus. In this pathway, activation of the NF-κB inducing kinase (NIK) upon receptor ligation led to the phosphorylation and subsequent proteasomal processing of the NF-κB2 precursor protein p100 into mature p52 subunit in a IKK1/IKKa dependent manner. Then p52 dimerizes with RelB to appear as a nuclear RelB: p52 DNA binding activity and regulate a distinct class of genes. In contrast to the canonical signaling that relies upon NEMO-IKK2 mediated degradation of IκBα, -β, -ε, the non-canonical signaling critically depends on NIK mediated processing of p100 into p52. Given their distinct regulations, these two pathways were thought to be independent of each other. However, recent
Introduction

analyses revealed that synthesis of the constituents of the non-canonical pathway, viz RelB and p52, is controlled by the canonical IKK2-IκB-RelA: p50 signaling. Moreover, generation of the canonical and non-canonical dimers, viz RelA: p50 and RelB: p52, within the cellular milieu are also mechanistically interlinked. These analyses suggest that an integrated NF-κB system network underlies activation of both RelA and RelB containing dimer and that a malfunctioning canonical pathway will lead to an aberrant cellular response also through the non-canonical pathway.

In immunity-

NF-κB is a major transcription factor that regulates genes responsible for both the innate and adaptive immune response. Upon activation of either the T- or B-cell receptor, NF-κB becomes activated through distinct signaling components. Upon ligation of the T-cell receptor, protein kinase Lck is recruited and phosphorylates the ITAMs of the CD3 cytoplasmic tail. ZAP70 is then recruited to the phosphorylated ITAMs and helps recruit LAT and PLC-γ, which causes activation of PKC. Through a cascade of phosphorylation events, the kinase complex is activated and NF-κB is able to enter the nucleus to upregulate genes involved in T-cell development, maturation, and proliferation.

In neurons-

In addition to roles in mediating cell survival, NF-κB has been demonstrated to have diverse functions in the nervous system including roles in plasticity, learning, and
memory. In addition to stimuli that activate NF-κB in other tissues, NF-κB in the nervous system can be activated by Growth Factors (BDNF, NGF) and synaptic transmission such as glutamate. These activators of NF-κB in the nervous system all converge upon the IKK complex and the canonical pathway (Karin and Ben, 2000).

Recently there has been a great deal of interest in the role of NF-κB in the nervous system. Current studies suggest that NF-κB is important for learning and memory in multiple organisms including crabs, fruit flies, and mice. NF-κB may regulate learning and memory in part by modulating synaptic plasticity, synapse function, as well as by regulating the growth of dendrites and dendritic spines.

Genes that have NF-κB binding sites are shown to have increased expression following learning, suggesting that the transcriptional targets of NF-κB in the nervous system are important for plasticity. Many NF-κB target genes that may be important for plasticity and learning include glutamate receptors (AMPA-R and NMDA-R), growth factors (BDNF, NGF) cytokines (TNF-alpha, TNFR) kinases (PKAα), and synaptic scaffolding proteins (PSD-95) (Ghosh and Karin, 2002).

Clinical significance

NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have misregulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from
Introduction

conditions that would otherwise cause it to die via apoptosis. Defects in NF-κB results in increased susceptibility to apoptosis leading to increased cell death. This is because NF-κB regulates anti-apoptotic genes especially the TRAF1 and TRAF2 and, therefore, checks the activities of the caspase family of enzymes, which are central to most apoptotic processes.

In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes); in addition, some tumor cells secrete factors that cause NF-κB to become active. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus, NF-κB is the subject of much active research among pharmaceutical companies as a target for anti-cancer therapy.

However, cautions should be excised for blockage of NF-κB activity as a broad therapeutic strategy in cancer therapy. Although convincing experimental data have identified NF-κB as a critical promoter of cancer development, creating a solid rationale for the development of antitumor therapy that suppresses NF-κB activity. On the other hand, compelling data have also shown that NF-κB activity enhances tumor cell sensitivity to apoptosis and senescence. In addition, it has been shown that canonical NF-κB is a Fas transcription activator and alternate NF-κB is a Fas transcription repressor. Therefore, NF-κB promotes Fas-mediated apoptosis in
Introduction

cancer cells, and thus inhibition of NF-κB may suppress Fas-mediated apoptosis to impair host immune cell-mediated tumor suppression.

Inflammation

Because NF-κB controls many genes involved in inflammation, it is not surprising that NF-κB is found to be chronically active in many inflammatory diseases, such as inflammatory bowel disease, arthritis, sepsis, gastritis, asthma, atherosclerosis and others. It is important to note that the key regulators of NF-κB are associated with elevated mortality, especially from cardiovascular diseases. Elevated NF-κB has also been associated with schizophrenia.

Non-drug inhibitors

Many natural products (including anti-oxidants) that have been promoted to have anti-cancer and anti-inflammatory activity have also been shown to inhibit NF-κB. Recent work by Karin and Ben (2000) and others has highlighted the importance of the connection between NF-κB, inflammation, and cancer, and underscored the value of therapies that regulate the activity of NF-κB. Extracts from a number of herbs and dietary plants are efficient inhibitors of NF-kappaB activation in vitro. The circumsporozoite protein of Plasmodium falciparum has been shown to be an inhibitor of NF-κB.
Introduction

As a drug target

Aberrant activation of NF-κB is frequently observed in many cancers. Moreover, suppression of NF-κB limits the proliferation of cancer cells. In addition, NF-κB is a key player in the inflammatory response. Hence methods of inhibiting NF-κB signaling have potential therapeutic application in cancer and inflammatory diseases. The discovery that activation of NF-κB nuclear translocation can be separated from the elevation of oxidant stress gives an important hint to the development of strategies for NF-κB inhibition. A new drug called denosumab acts to raise bone mineral density and reduce fracture rates in many patient sub-groups by inhibiting RANKL. RANKL acts through its receptor RANK, which in turn promotes NF-κB. RANKL normally works by enabling the differentiation of osteoclasts from monocytes. Disulfiram, olmesartan and dithiocarbamates can inhibit the nuclear factor-κB (NF-κB) signaling cascade. Anatabine alleged anti-inflammatory effects is claimed to result from modulation of NF-κB activity.

In silico proteomics

Proteomics is a tool for the simultaneous determination of the protein composition of complex samples. Computational modeling is useful as a means to assemble and test what we know about proteins and networks. Models can help address key questions about the measurement, definition and function of proteomic networks. Current understanding of the proteins, interactions and pathways that comprise
Introduction

signaling networks is detailed, yet it remains incomplete. Recent experimental techniques for unraveling intricate signaling networks have become increasingly quantitative and multiplex. New approaches are now needed to compile the existing quantitative biological knowledge and to maximize the information extracted from large-scale signaling and proteomic datasets. Computational models formalize a complex biological or experimental process mathematically, which can be useful for assembling and analyzing quantitative data (Kevin and Douglas, 2006). Modeling is thus critical for fields such as proteomics, genomics and systems biology.

Chemically, a protein is a complex molecule, composed of carbon, hydrogen, oxygen and usually, sulphur. Some proteins also incorporate phosphorus, iron, zinc and copper. These basic elements combine into molecules called amino acids, which are the building blocks of proteins. It was believed that protein identification could be achieved based on the accurate measurement of the protein MW. But, it was quickly realized that the growing size of databases and the accuracy of the mass measurement limited the unambiguous identification of proteins based on their MW.

There has been a lot of confusion in the field of proteomics on the issue of the complete proteome coverage and low-abundance proteins. This confusion is, in part, due to a bit of propaganda about the power of the technology, as well as some confusion related to the definition of proteome and proteomics. The idea of the comprehensive study of the proteome is more an idealism than a reality (Daniel, 2005). Modelling programs can help predict how a protein will fold under various
Introduction

conditions. Using this information, researchers can learn the shape of each protein and get an idea of what it does.

Docking is an automated procedure for predicting the interaction of ligands with biomacromolecular targets. The motivation for this work arises from problems in the design of bioactive compounds, and in particular the field of computer-aided drug design. Progress in biomolecular x-ray crystallography continues to provide a number of important protein and nucleic acid structures. These structures could be targets for bioactive agents in the control of animal and plant diseases, or simply key to understanding of a fundamental aspect of biology. The precise interaction of such agents or candidate molecules is important in the development process. The traditional approach for targeting an enzyme is through the development of inhibitors of the enzyme's catalytic activity. There has been some level of success with this approach starting with analogues of the potent natural product Staurosporine with PKC (Martinbyarmon et al., 1993). In addition to this, complex natural products have provided many of the initial insights into the structural features required for ligand binding and for the diversity of biological responses. Docking studies with the program AUTODOCK (Morris et al., 1998) probably gave an insight into the binding modes of these inhibitors.

Auto Dock uses three search methods (a genetic algorithm, a local search method and an adaptive global-local search method based on Lamarckian genetics (LGA)) in
Introduction

conjunction with an empirical force field that allows the prediction of binding free energies for docked ligand.

Understanding the proteome – the full set of proteins for an organism – is a daunting task, but the potential rewards are great. Most modern medicines, for example, are either proteins or work by binding to proteins. In medicine, a better understanding of how proteins work could lead to the development of target based drug. Also, characterisation of the interaction between flavonoids and their target sites could potentially allow the design of second-generation inhibitors. However, the chief challenge facing is the lack of efficient therapeutic modalities available. For the foreseeable future drug development may remain a major focus of ongoing research. Overall, prospective bioinformatics studies on NF-κB and inflammation are scarce, limiting our knowledge about the exposure-disease relation. Despite this caveat it is anticipated that bioinformatics approaches may significantly contribute to the management of inflammation in the future.
Objectives
Objectives of the research work

- To study the proteomics of NF-κB sequence retrieved from different organisms with reference to its properties and function.
- To do the comparative sequence analysis and build evolutionary history.
- To compare the structural similarity of the sequences.
- Docking studies of the individual sequences with the standard drugs.