Biology

Targets and Mechanisms

A new role for ubiquitination in the activation of NF-κB

Ubiquitination, the attachment of a chain of ubiquitin molecules, is known to have a major role in the activation of NF-κB by way of marking its inhibitor, IkB, for proteolytic degradation. Recently, Kun-Liang Guan and co-workers report evidence for an additional function of this type of covalent modification in the NF-κB signal transduction pathway [1,2].

The authors found that IKKγ, also known as NEMO, becomes ubiquitinated when HeLa cells are stimulated with TNFα. IKKγ is a non-catalytic component of the IkB kinase (IKK) complex, which phosphorylates IkB in response to a variety of proinflammatory stimuli and thereby triggers its ubiquitination and subsequent degradation by the proteasome.

IKK has, in recent years, received considerable attention within the pharmaceutical industry as a target for the discovery of anti-inflammatory drug candidates. In contrast to its effect on IkB, ubiquitination of IKKγ appears to result in the activation of IKK rather than the degradation of this regulatory subunit. The protein responsible for IKKγ ubiquitination was identified in this study as c-IAP1, an inhibitor of apoptosis, which is part of the TNF receptor complex and acts as ubiquitin ligase.

Furthermore, the authors propose that IKKγ ubiquitination stimulates IKK phosphorylation in the activation loop of the catalytic domain. If correct, ubiquitination as a means to regulate protein activity rather than turnover joins phosphorylation and acetylation in the control of NF-κB.

1 Tang, E.D. *et al.* (2003) A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. *J. Biol. Chem.* 278, 37297–37305

2 Chen, L.F. and Green, W.C. (2003) Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. *J. Mol. Med.* 81, 549–557

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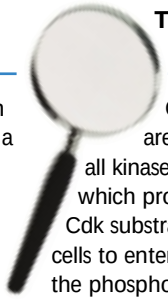

The hard task of identifying protein kinase substrates

Cyclin-dependent kinases (Cdks) are probably the most studied of all kinases, however, we do not know which proteins make up the repertoire of Cdk substrates. To know how Cdk1 causes cells to enter mitosis, one must know how the phosphorylation status of a protein alters its function. To do this, one must first identify the proteins that are phosphorylated.

Ubersax and colleagues have studied *Saccharomyces cerevisiae* [3], in which Cdk1 (formerly Cdc28) is the only Cdk required for the cell cycle, and modified the coding sequence of *Cdk1* gene so that the ATP pocket can now accept an ATP analogue that that is not recognized by other protein kinases. This permits the detection of specific Cdk1 substrates. The researchers then performed an analysis in cell extracts of 385 ORFs from the yeast genome that encode a Cdk1 phosphorylation consensus sequence to test the system.

Previously identified Cdk1 substrates, such as Cln2 (cyclin B), were detected, which indicated that this approach should detect other Cdk1 substrates. In total, 695 ORFs were tested and 181 candidate substrates were identified. Interestingly, 123 proteins with a complete consensus site were not detected by this approach, hinting that genome mining will probably overestimate the number of potential substrates. In a next step, the authors confirmed protein phosphorylation by the mutated form of Cdk1 *in vivo* by examining mobility shifts on protein gels. In their example, Slk19, a substrate of separase, was shown to be phosphorylated by Cdk1.

The task of substrate identification becomes even more daunting when one realises that this information is required for the 518 protein kinases present in human cells. But without this information, our understanding of how protein kinases participate in cell function will always be incomplete.

3 Ubersax, J.A. *et al.* (2003) Targets of the cyclin-dependent kinase Cdk1. *Nature* 2003 425, 859–864

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Genomics and Proteomics

Essentially conserved: understanding essential genes

The promise of killing an organism by disabling a single gene product is an attractive one for drug discovery researchers. Recently, Gerdes and coworkers published the results of an extensive essentiality screen in *Escherichia coli* MG1655 [4], which represents an interesting and informative step forward.

Large-scale transposon mutagenesis generated insertions at 18,000 distinct locations and points of insertion were identified with nested PCR. Because transposon insertions (TIs) in essential genes should cause a lethal phenotype, any gene free of TIs after the screen can be roughly characterized as essential. Good coverage is required for this technique to work – and the authors have it, with 3.2 inserts per kb of genomic sequence.

Of the 4291 protein-coding genes in MG1655, 87% were assessed and 14% were found to be essential. Several results are striking: first, groups of proteins involved in nucleic acid and protein metabolism contain a higher percentage of essential genes (28% and 48%, respectively) than the genome overall. Second, <180 broadly conserved genes account for over a quarter of essential genes identified in this screen; these might represent a conserved 'core' set of required genes shared by most bacterial species. Finally, roughly 30% of essential genes are unique to *E. coli* and its closest relatives and, of these, >75% encode proteins of unknown function.

Identification of promising targets is central to antibiotic drug discovery and essential genes remain the low-hanging fruit. Gerdes *et al.* make an attempt to illuminate what might be a core, crucial gene set of many pathogenic species.

4 Gerdes, S.Y. *et al.* (2003) Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. *J. Bacteriol.* 185, 5673–5684

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Comparative genomics is going to the dogs

Recently, *Science* reported the first draft genome sequence of the dog [5]. Comparing genomes from different mammalian species might ultimately help us to understand what makes us 'human'.

For instance, the genome sequence of our closest animal relative, the chimpanzee, when available, could help us understand what specifically shapes the human brain. After all, the human and monkey genomes differ by 'only' a few percent, or 5–7 million years of evolution. The trouble is, those few percent still amount to over a hundred million mutations, and most are in genes that are totally irrelevant to what we would really like to know.

It would be helpful to compare organisms that have minimal genetic differences yet differ maximally in body plan – which brings us back to dogs. Therefore, identifying the genetic basis of what distinguishes between different dog species will probably provide us with new set of crucial genes that define the mammalian body plan. These genes might not be particularly interesting, but knowing their gene families could enable us to fill the knowledge gap between gene sequences *per se* and what the genes ultimately code for.

The Human Genome Project has often been sold as a 'free lunch' for drug discovery. However, before we can begin to understand disease and find drug targets in a truly rational way, a huge increase in our basic understanding of biology is essential. And, yes, the availability of the human, and other, genomes could ultimately be of some help in achieving all this, perhaps along with a little help from our (best) friends!

5 Kirkness, E.F. *et al.* (2003) The Dog Genome: Survey Sequencing and Comparative Analysis. *Science* 301, 1898–1903

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Small molecule phage display

Woiwode *et al.* [6] have recently demonstrated the successful combination of phage display with the screening and encoding of small molecules that can modulate important biological function as monitored by cell-based assays. The method is based on a DNA-based encoding system whereby a particular DNA sequence tag is assigned to a specific small molecule.

The authors used filamentous and T7 phages to demonstrate the proof-of-concept. The authors further demonstrated the use of the approach by synthesizing derivatives of folate, to select for compounds that can internalize the phages.

Typically, 0.005–0.01% of input phage were recovered with a tenfold difference over background, which represented phage recovered from cells incubated in the presence of folate. Radiolabelled probes corresponding to the tags initially bound strongly to 9 of the 20 clones representative to phage displaying high-affinity folate analogues. After quantitative analysis, an additional five clones corresponding to part of the 20 high-affinity folate analogues were identified based on signal-to-noise ratio exceeding twice the standard deviation from the average of all 960 tested clones.

This paper extends the advantages of phage display and combinatorial chemistry towards small-molecule drug discovery. One can envision automation of the process to facilitate applications to larger libraries. Further applications of the method with diversity-oriented chemical synthesis will lead to larger chemical libraries that can rival the complexity of biological libraries, such as naïve human antibodies.

Extending the advantages of phage display to combinatorial small-molecule chemistry would revolutionize small molecule discovery.

6 Woiwode , T.F. *et al.* (2003) Synthetic compound libraries displayed on the surface of encoded bacteriophage. *Chem. Biol.* 10, 847–858

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Cancer Targets and Mechanisms

A link between cancer and Alzheimer's?

Drug discovery efforts for Alzheimer's disease (AD) have aimed at blocking production of the amyloid-β peptide (Aβ), produced from the amyloid precursor protein that is cleaved by secretase enzymes, by inhibiting BACE or the γ-secretase complex, namely presenilin 1.

At present, several γ-secretase inhibitors have been identified that can abolish Aβ production. However, these compounds have toxic side-effects, relating to the inhibition of Notch-1, which has led researchers to explore alternative ways to block Aβ production without interfering with the Notch pathway [7].

One approach included looking at the ATP requirement of Aβ production in mouse neuroblastoma (N2A) cells overexpressing mutant APPswe and PS1 DeltaE9 proteins, where abundant Aβ is produced. One inhibitor was found; STI571 or Gleevec, which is a selective tyrosine kinase inhibitor that binds to the ATP-binding sites of several tyrosine kinases, including Abl.

In the N2A stable cell lines overexpressing the APPswe and the PS1 DeltaE9 mutants, treatment with Gleevec inhibited Aβ-40 and -42 peptide production, 50–60%, respectively. Also, Gleevec could inhibit Aβ production in N2A cells overexpressing the C99 substrate, which suggests it is inhibiting Aβ production through γ-secretase activity.

The ability of Gleevec to inhibit Aβ was independent of Notch cleavage, which argues that it can reduce Aβ formation without having an affect on the Notch pathway. This study also demonstrated that the reduction in Aβ levels did not depend on the Gleevec target Abl.

The discovery that Gleevec can provide protection against the primary cause of AD suggests that it could slow or stop progression of the disease. These results suggest that a kinase activity other than Abl could be the target of Gleevec, and have reinvigorated a largely ignored area of kinase research and AD.

7 Netzer, W.J. *et al.* (2003) Gleevec inhibits β-amyloid production but not Notch cleavage. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12444–12449

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In search of CD155 function

CD 155, originally identified as the receptor for human poliovirus, has also previously been shown to be overexpressed in colon tumours. Based on the gene organization of *CD155*, four isoforms have been described: two that code for membrane bound forms (CD155-α and -δ) and two soluble forms (CD155-β and -γ). However, the existence of the soluble isoforms has not previously been demonstrated.

Now, Baury *et al.* demonstrate the existence of soluble CD155 (sCD155) isoforms, *in vitro* and *in vivo* [8]. sCD155 were shown to be present in conditioned culture media from two colon cancer cell lines and can also be recovered from the culture media from gastric, breast, prostate and melanoma cancer cell lines.

ELISA was used to estimate the amount of sCD155 in 58 samples of human serum (1.04 nM) and 21 samples of cerebrospinal fluid (0.03 nM). More importantly, the authors demonstrated that sCD155 is generated by secretion of the isoform into the culture media and not of proteolytic cleavage.

This paper describes a comprehensive distribution of transcripts in different cell types. Earlier studies indicating overexpression of CD155 in colon tumours and the low level of sCD155 in serum suggest a potential antibody payload approach to treat colon cancer in light of the low level of expression in normal colon cells.

However, the high transcript expression level of the CD155-α isoform in liver and other organs raises some concern. These findings imply an important role of CD155 in normal tissues but some issues require clarification, such as the distribution of the different CD155 isoforms through immunohistochemistry from a wider panel of normal and diseased tissues, especially tumours. This might provide some clues to elucidate the cellular role of CD155 and its potential as a therapeutic target in cancer.

8 Baury, B. *et al.* (2003) Identification of secreted CD155 isoforms. *Biochem. Biophys. Res. Commun.* 309, 175–182

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Viral Targets and Mechanisms

Analytical screening of new and developmental HIV1 antivirals

A recent study [10] contextualizes microplate technology in terms of a relevant area of high interest, namely, HIV1 and drug targeting. This study looks closely into inhibitors of the retrovirus and presents a pertinent study of viral anti-proliferatives that can be assessed by microplate platform technology.

HIV1 replication is based on reverse transcriptase (RT) activity that is associated with RNA and DNA-dependant DNA polymerase and ribonuclease H (RNase H) activity. RT activity can therefore be targeted as a means of producing noninfective virons.

Parniak *et al.* discuss the relative lack of work in the RT-RNase H drug-targeting area. Outside of the microsystems arena, many currently favoured analytical techniques are labour intensive, cumbersome and inadequate in terms of cost, required technical support, applicability to HTS and use of materials. The fluorescence assay detailed in this paper for measurement of RNase H activity provides an inexpensive and rapid means of antiviral drug testing.

Volumes of 100 μl of 50 nM solutions are employed and the experimenters were seeking good temperature stability, a low baseline fluorescence signal and a moderate to low melting temperature. Other optimization parameters included linearity of reaction over the time frame of the experiments with coefficients of variation in the order of 4–5%.

Parniak *et al.* observed that up to three runs could be completed in one day providing a throughput of 7000+ compounds per day. The fluorescence assay described in this paper is ideally suited for rapid quantitative measurements and for validated drug screening with the use of robotic systems. This, and associated methods, will undoubtedly provide a powerful tool in screening of extensive libraries of current and newly synthesized antivirals.

10 Parniak, M.A. *et al.* (2003) A fluorescence-based high-throughput screening assay for inhibitors of human immunodeficiency virus-1 reverse transcriptase-associated ribonuclease H activity. *Anal. Biochem.* 322, 33–39

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AP2γ and a transgenic model of gene amplification in breast cancer

Evidence suggests that the transcription factor AP2γ is overexpressed in breast cancer. In addition, AP2γ appears to regulate the expression of ErbB2, a receptor tyrosine kinase associated with poor prognosis. However, the precise role of AP2γ in tumourigenesis has yet to be established.

Lack of AP2γ results in lethality during early embryogenesis, partly due to reduced proliferation of placental cells, suggesting that AP2γ is required for cellular proliferation and might therefore be an oncogene. To test this hypothesis, researchers at the University of Bonn Medical School (http://www.meb.uni-bonn.de) have generated a model for AP2γ amplification in breast cancer [9].

In female mice, over-expression of AP2γ resulted in a failure in lactation, which was found to be cell autonomous and could not be rescued by the addition of lactogenic hormones. At the molecular level, overexpression of AP2γ resulted in induction of markers of proliferation and apoptosis, including the pro-apoptotic factor IGFBP5, and suppression of genes that are characteristic of the differentiated state.

Therefore, it appears that AP2γ can promote proliferation and apoptosis at

the expense of differentiation and could act as a switch between cellular states. However, what is not clear from the current study is which of these functions of AP2γ is most pertinent to the aetiology of breast cancer. As ectopic expression of AP2γ was not sufficient to induce mammary tumours in the transgenic mice, it is unlikely that AP2γ initiates breast cancer. Instead, a role in tumour progression is more probable. This transgenic model will doubtless prove a valuable tool with which to further dissect the role of AP2γ in this complex process.

9 Jager, R. *et al.* (2003) Transcription factor AP2γ stimulates proliferation and apoptosis and impairs differentiation in a transgenic model. *Mol. Cancer Res.* 1, 921–929

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Business

Patents

'Tense' matters in patent applications

The recent opinion issued by the Court in the Hoffmann-La Roche (http://www.roche.com) versus Promega (http://www.promega.com) case is an eye-opener to the handling of patent examples in patent applications [1]. In the case referred, Roche had a prophetic example (experiment that was not performed) written in past tense. The court held that drafting an example in past tense, when it has not been performed, misleads the reader and thus amounts to inequitable conduct. In some circumstances, inequitable conduct is adequate to invalidate a patent.

Patent applications should minimally consist of a specification including the title, abstract summary and illustrations, if necessary, and at least one claim. Patent examples are drafted as a part of the specification and often aid in describing given experimental data associated with the invention. The patent example section is not an absolute requirement for filing an application. However, for biotechnology applications, it has been useful in rendering use and enablement support for the claimed invention. As far as the language goes, patent examples are described in the past tense while describing the experiments performed. It is often a practice in the patent world to describe the experiments 'to be done' under patent examples. These examples are written in the present or the future tense and are called as prophetic or paper examples. Describing the prophetic example(s) in the original application helps later in the prosecution to introduce the data in the form of a declaration.

The art of writing patent applications is constantly evolving. However, there are guidelines that are given in the Manual of Patent Examining Procedure (MPEP) that the patent examiners refer to. MPEP clearly states how the examples should be drafted in a patent application. The lesson learnt here is that caution needs to be exerted when describing prophetic examples. The authors give some helpful hints on how scientists and practitioners can make a concerted effort in order to avoid such mistakes.

1 Potter, J.E. and Talukder, G. (2003) Past versus present: the importance of tense in patent application examples. *Nat. Biotechnol.* 21, 1397–1398

What next for research tool patents?

Often, landmark discoveries related to drug candidates make big news, however, lost in the glory of the new drugs are the research tools that are used in discovering the drug targets [2]. If patented, these tools are important sources of revenue for academia and biotechnology companies.

However, research tool patents have been a challenge to enforce. This is partly due to the 'research exemption' rule detailed in Section 271 (e) (1) of Title 35 of the United States Code (USC), which enables the generic drug makers to get regulatory approval before the drug patent expired. However, the breadth and scope of 'research exemption' rule has served as a 'safe harbour' for many drug discovery efforts, making it difficult for research tool patent holders to prove infringement.

Raubicheck *et al.* [x] review the renewed value of research tool patents in the wake of the Integra versus Merck decision. The Integra (http://www.integra-ls.com) patent claims compositions and method relating to an RGD peptide that regulates cell adhesion. Scientists from the Scripps Research Institute (http://www.scripps.edu) under a license agreement with Merck (http://www.merck.com) used the patented peptide for identifying novel antiangiogenic drug candidates; Integra argued that Merck infringed the peptide patents by using the claimed peptides. Merck defended that the drug-screening activities were exempt from liability for infringement under USC 271 (e) (1), because they were 'reasonably related to' information required by the FDA. The Court ruled that the use of the patented peptide in the early stage drug-screening activity does not directly lead to clinical testing required by FDA and thus can not be sheltered under research exemption.

Although more clarity is required in terms of defining exemption, the Integra decision partially salvages the 'value and glory' of research tool patents. However, on the issue of 'value', damage analysis and reasonable royalty estimates could be particularly problematic for research tool patents, which by themselves might have no market value. Thus, it might be prudent for patent owners or research tool users to consider the 'value' of the patented tool carefully before considering litigation or licensing.

1 Raubicheck, C. *et al.* (2003) Integra versus Merck: a mixed bag for research tool patents. *Nat. Biotechnol.* 21, 1099–1101

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