Cell signaling: signal transduction and gene transcription

This report addresses some highlights of NMHCC's *2nd Annual International Conference on Cell Signaling: Signal Transduction and Gene Transcription*, held in San Diego, CA, USA, during July 1998. The conference presentations were of a high standard overall, and the participants were regularly seen in small groups discussing the day's science. I apologize to presenters of some of these excellent talks that cannot be described due to space limitations. The first morning was devoted to new drug discovery technologies, and the afternoon to some of the new signal transduction modulating drugs under investigation.

Split hybrid system

Phyllis Goldman (ICOS, Seattle, WA, USA) discussed the 'split hybrid', a variant of the yeast two hybrid system. Two interacting protein domains of interest are fused to the LexA DNA-binding domain and the VP16 transactivating domain as usual, but instead of the *LexA* operon being spliced into the promoter region of the gene of interest, it is put into the promoter region of the *Tet* (repressor protein) gene. The *Tet* operon is put upstream of the histidine synthase 3 (*HIS3*) gene transfected into $HIS3^{-/-}$ yeast, which are then cultured without histidine. These cells can only grow if the transcription of the Tet repressor is blocked by inhibition of the two hybrid protein–protein interaction, thus providing positive selection of the inhibitory activity. The *Tet* operon is also put upstream of the *LacZ* gene to act as a reporter. The system can be fine tuned to very low background and high sensitivity by addition of tetracyclin and 3-aminotriazole, which suppresses endogenous signal leakage. The system has been used to clarify important amino acids in the cAMP-response-element binding protein (CREB)–CREB-binding protein interaction and is now being used to screen for both protein and small-molecule inhibitors of protein–protein interactions.

Artificial zinc fingers

Casey Case of Sangamo Biosciences (Aurora, CO, USA) described a technique for creating artificial zinc fingers that bind to specific DNA sequences using phage libraries and a reiterative synthetic system based on rules for DNA-base selectivity for zinc-finger binding. These modular proteins are strung together and repressor or activator domains are added to make artificial transcription factors for incorporation into viral vectors for gene therapy. Current uses include targeting the KRAB repressor both to the hypoxia-inducible factor 1 (HIF-1) response element in the vascular endothelial growth factor (VEGF) promotor, to block angiogenesis in hypoxic tumors, and to the CCR5 (chemotactic cytokine receptor) promoter to prevent HIV infection of immune cells.

Nuclear hormone receptor ligands

David Moore's (Baylor, New Orleans, LA, USA) talked on finding ligands for orphan nuclear hormone receptors (NHRs). In searching for the endogenous, and presumably ubiquitous, ligand for the orphan NHR – CAR (constitutive activator of retinoid response), which dimerizes with the RXR receptor – the discovery was made that CAR not only binds two androgens (5α-androst-16-en-3α-ol and 5α-androstan-3α-ol) that show no affinity for the androgen receptor, but that it is also deactivated by them. To find out whether these are accidental antagonists or whether the system is really constituitively active, until deactivated by ligand, mutations were made in the ligand-binding domain (LBD). These abolished androstanol binding completely, but left the receptors fully active, which strongly suggests that a new form of NHR regulation exists – i.e. deactivation by an endogenous ligand.

X-ray crystallography

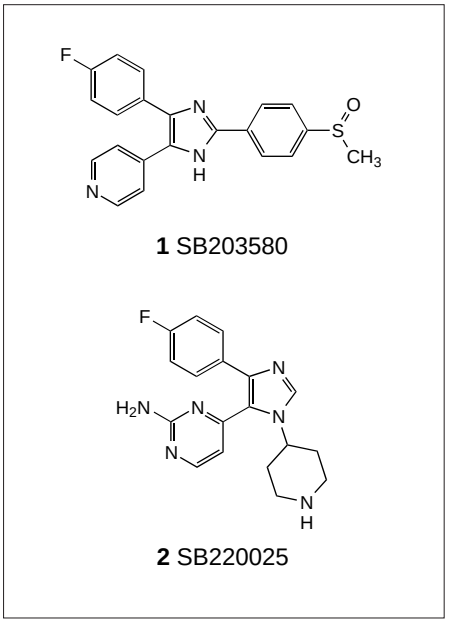
Betsy Goldsmith (SW Medical Center, Dallas, TX, USA) compared the crystal structures of unactivated Erk2 with the activated form, diphospho-Erk2 (p-Thr183, p-Tyr185). There proved to be a much smaller difference between them than had been anticipated. The p-Tyr phosphate group is buried within the protein, and the Tyr phenyl group actually makes up a portion of the substrate-binding proline specificity pocket of Erk2. The Erk2 protein has a long C-terminus that follows the kinase domain; it projects out of the C-terminal lobe in a long strand behind the protein forming a fairly large α-helix (α_{L16}), which is attached to the back of the N-terminal domain. Close examination of the diphospho-Erk2 structure reveals the purpose of this appendage. In the active form it provides both a structural salt bridge to the activation lip and a dimerization surface for Erk2. At first it was not obvious if this was simply a packing artifact, but biochemical experiments have shown that diphospho-Erk2 forms strong dimers, and that it is these dimers that enter the nucleus.

Goldsmith then introduced us to the 'star' molecule of the meeting – the p38α/β inhibitor SB203580 (**1**). Other presentations by John Lee (SmithKline Beecham, King of Prussia, PA, USA), Stephen O'Keefe (Merck, West Point, NJ, USA) and Frank Salituro (Vertex, Cambridge, MA, USA) were on the same theme, and will be discussed together. The X-ray structures of the inhibitors **1** and **2** show several features common to most ATP-competitive kinase

  1359-6446/98/$19.00. PII: S1359-6446(98)01247-1

inhibitors, such as a backbone hydrogen bond in the hinge region, in this case from the pyridine nitrogen to the amino group of Met109, and also the imidazole imino group is involved in hydrogen bonding.

Molecule **1** has an electron rich Tyr aromatic stacking above the electron deficient 2-(4-methylsulphinylphenyl) ring, and in inhibitor **2**, in which this is missing, the N1-(4-piperidinyl) nitrogen is protonated and forms a salt bridge with Asp168. Most interestingly, the fluorophenyl ring sits in a hydrophobic pocket partly created by a close interaction between the fluorine atom and Val105 and partly by a 120° rotation of the Thr106 side chain, which also allows the threonine hydroxyl group to contribute to an extant hydrogen bonding network. Site directed mutagenesis showed this residue to be vital for inhibitor binding. Mutating Thr106→Met, as seen in the **1**-insensitive γ and δ isoforms, made p38α insensitive to **1** and **2**. Mutation of the corresponding residues in p38γ, p38δ, JNK1 and Erk2 to Thr in each case made the enzymes considerably more susceptible to the inhibitors. Compound **2** has enough Erk-2 binding to be cocrystallized with it. Very satisfyingly, the only major difference seen (for a 1000-fold loss of IC_{50}) was the replacement of Thr106 with Gln105, partially filling the hydrophobic binding pocket. Thus, for the first time, pharmacologically useful differences in protein binding in kinase inhibitors have been satisfactorily explained by a single residue change.



IκB kinases and the p70 S6 kinase

Frank Mercurio (Signal Pharmaceuticals, San Diego, CA, USA) and John Blenis (Harvard, Cambridge, MA, USA) discussed the IκB kinases and the p70 S6 kinase, respectively. Two IκB kinases, IKK-1 and IKK-2, have been found. They are activated within 5 min of stimulation and deactivated within 30 min. They phosphorylate the regulatory serines on IκB-α and IκB-β, leading to IκB polyubiquitination and degradation by the proteosome, which allows the liberated NF-κBs to enter the nucleus. They are substrates for an inflammation-activated MAPK (mitogen-activated protein kinase), MEKK-1, but from this point on nothing else is straightforward. The two IKKs can homo- and heterodimerize and both normally exist as a part of a 700 kDa 'signalosome'. Both are activated by radiation, but the human T-cell leukemia virus type 1 (HTLV-1) Tax protein only activates IKK-2, yet both stimuli activate NF-κB. Antibodies to the MAPK phosphatase MKP-1 recognize IKK-1/2 heterodimers but not IKK-2 homodimers. From such immunoprecipitates, the first further member of the signalosome was found, named IKKAP-1 – a 50 kD IKK-2 (not IKK-1) binding protein. This protein is required for IκB phosphorylation, and itself contains an Lzip dimerization domain. IKK-1 does not appear to be a good IκB kinase, but both IKKs do phosphorylate p65 Rel.

Regulation of $p70^{S6K}$, which activates translation by phosphorylation of the S6 ribosomal protein, is also extremely complex. Looking downstream of PI3K, Blenis' group first demonstrated that activation of the small Rho-like GTPase CDC42 with the pleckstrin-homology-containing guanine-exchange factor Dbl leads to $p70^{S6K}$ activation. A dominant negative CDC42 blocked $p70^{S6K}$ activation, whereas a constitutively activated CDC42 activates $p70^{S6K}$. However, the active mutant could not activate $p70^{S6K}$ in the presence of wortmannin [a phosphoinositide 3-kinase (PI 3-kinase) inhibitor], suggesting that a second PI 3-kinase-sensitive activator is needed. Protein kinase Cζ (PKCζ) proved to be one such activator, phosphorylating three sites in the catalytic domain. This still did not lead to an active kinase and the crucial activation loop residue Thr229 turned out to be a substrate for yet another PI 3-kinase-activated kinase, namely p21-dependent kinase 1 (PDK-1, which has also been implicated in Akt/protein kinase B activation, and is a hot prospect for the next kinase *du jour*). PDK-1 phosphorylation alone activates $p70^{S6K}$ threefold, and with PKCζ phosphorylation this rises to a sevenfold activation. However, none of this can occur until four Erk-mediated phosphorylations in the C-terminal pseudosubstrate have occurred. The casein kinase II (CKII) phosphorylation site in the N-terminus is currently of unknown function. Overall, it is probable that a membrane complex containing (at least) CDC42, PDK-1, PKCζ, $p70^{S6K}$ and an unknown adaptor protein is involved in the pathway.

Phosphatase lethargy

Nick Tonks (Cold Spring Harbor Laboratory, ME, USA) gave some persuasive reasons to encourage the industry out of its 'phosphatase lethargy' – although seasoned observers think that somebody else's 10 nM lead or the traditional Merck benediction may be required as well. Using his inactivated, substrate-binding mutant phosphatase 'substrate traps', Tonks demonstrated that the closely related phosphatases, PTP1B and TCPTP have different and very narrow substrate specificities, and that these specificities also vary for differentially localized splicing variants of the same gene product. His point is that there are a host of phosphatases and that, *in vivo*, it is likely that most, like the kinases, have a very limited substrate range and can therefore be targeted for specific diseases. He also examined the tumor suppressor PTEN.

This phosphatase only dephosphorylates extremely acidic phosphopeptides; its real physiological role is probably deactivation of phosphatidyl-3,4,5-triphosphate (PIP_3) by removal of the 3-phosphate group. One naturally occurring oncogenic point mutant, Gly129→Glu, turns out to retain protein kinase activity, but not lipid kinase activity. In further support of this role, PTEN transfection into U87MG glioblastoma cells leads to deactivation of PKB and prevention of BAD (a pro-apoptotic member of the BCL-2 family) phosphorylation, both steps known to be survival-promoting, and downstream of PI3K. Transfection of the wildtype PTEN into LnCAP cells led to apoptosis, but the Gly129→Glu mutant did not. The structural basis for the selective ablation of lipid kinase activity awaits next year's conference.

Alexander J. Bridges
Parke-Davis
Pharmaceutical Research
Division of Warner-Lambert
Corporation
2800 Plymouth Road
Ann Arbor, MI 48105, USA
tel: +1 734 622 7103
fax: +1 734 622 3107
e-mail: Alexander.Bridges
@aa.wl.com

Imidazol receptors – an enlightening experience

For many pharmacologists, like myself, who trained more than a decade ago, the emergence of imidazoline receptors has been an enigma, and even those pharmacologists who have attempted to rectify their ignorance by reference to standard textbooks have been thwarted, as these receptors rarely, if ever, receive a mention. I was therefore extremely pleased to be able to attend a state-of-the-art symposium entitled *Identification, Characterisation and Controversies on the Role of Imidazol Receptors* during the recent International Congress of Pharmacology (München, Germany), in which six speakers actively working in the field presented a concise overview of the subject and its therapeutic potentials.

Historical perspective

The first speaker, Pascal Bousquet (Faculte de Medecine, Strasbourg, France), outlined the history of the imidazoline receptor. Initially it was known that the imidazoline compound clonidine, known to be an agonist of α_2-adrenoceptors, was able to act in the brainstem to induce hypotension. It then became apparent that clonidine was binding to receptors other than the α_2-adrenoceptors. These receptors were named imidazoline receptors (I-receptors). Bousquet defined such receptors as being 'receptors that are sensitive to imidazolines, but non-sensitive to catecholamines'. As early as 1992 it was recognized that there are several subgroups of imidazol receptors, the first classification being based on the relative affinities for clonidine and idazoxan (an α_2-adrenoceptor antagonist). The I_1-receptors have affinity for both clonidine and idazoxan, whilst I_2-receptors have affinity for idazoxan alone. More recently it has been postulated that both I_1- and I_2-receptors are subdivided into high- and low-affinity subtypes.

The high-affinity I_1-receptor has now been purified and has been shown to be distinct from any other known receptor. It has been identified in human brain stem and has been shown to be involved in the cardiovascular effects of clonidine-like drugs. There is, however, dispute about whether α_2-adrenoceptors are also involved in the cardiovascular effects of clonidine. Using the novel I_1-selective agonist LNP509 (patent pending), it has been shown in anaesthetized rabbits that both LNP509 and α_2-adrenoceptor agonists have only small effects on blood pressure, but a combination of the two agents results in a large reduction in pressure. This is taken to indicate that α_2-adrenoceptors are required for the effects of LNP509.

Imidazoline analogues and hypertension

Rilmenidine is one of a new class of drugs that are analogues of imidazoline and have been developed for the treatment of hypertension. In humans, rilmendine has been shown to be effective in the relief of all forms of hypertension, whilst being free from the common side effects of sedation, drowsiness, dry mouth and weight change. Furthermore, this compound has been shown to be effective in preventing bicuculline-induced arrhythmias in anaesthetized rabbits, at sub-hypotensive doses. The results therefore suggest that rilmenidine may be a useful antihypertensive and/or anti-arrhythmic agent, relatively free from adverse effects.

The controversy within the field was then highlighted by the presentation of Bela Szabo (Universität Freiburg, Germany). Using conscious rabbits he has shown that the effects of rilmenidine and moxonidine (both I_1-receptor agonists) and UK14304 (an α_2-adrenoceptor agonist) can all be reversed by yohimbine (an α_2-adrenoceptor antagonist). Similar results were found using the α_2-adrenoceptor antagonist SKF86466. These results suggest that rilmenidine and moxonidine both produce their effects via α_2-adrenoceptors.

  1359-6446/98/$19.00. PII: S1359-6446(98)01243-4