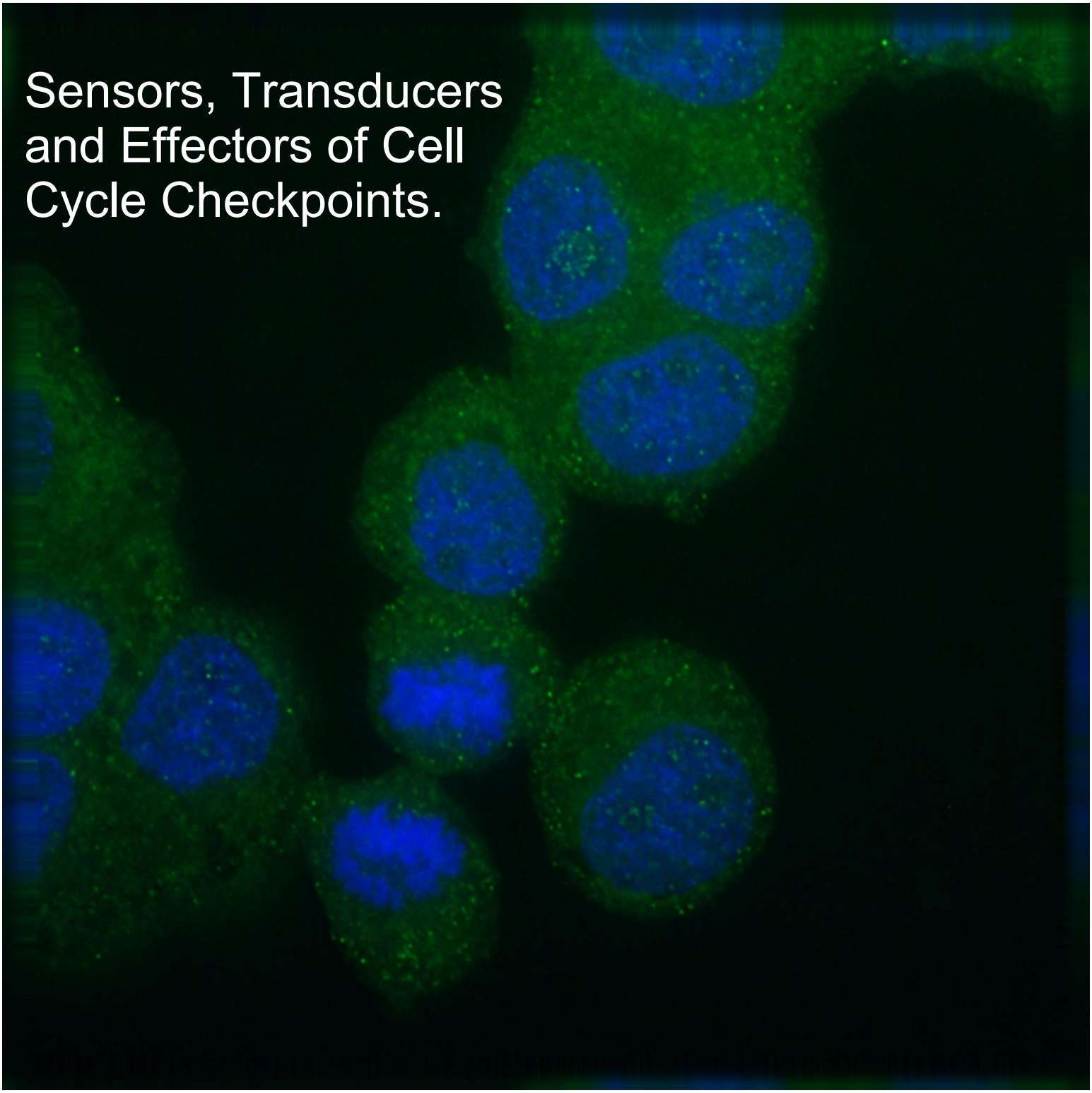
Annual RUR
2002, Volume 1
Reviews in Undergraduate Research
International Journal of Excellence in Undergraduate Research

Sensors, Transducers and Effectors of Cell Cycle Checkpoints.



Also in this issue: CP Violation, MAP Kinases, Jupiter's Orbit, Genetic Engineering, Condensed Matter Physics, and Caveolae.

Annual RUR

Letter from the Editor & Staff

Dear Reader,

When we began this journal last spring we had the needs of one group in mind: Undergraduates. We felt that there was a large gap in scientific literature which failed to meet the needs of an undergraduate student. College students usually have taken several introductory courses within their major and thus might find articles in popular scientific journals such as *Scientific American* a bit too basic. However, as students only beginning to learn the vocabulary and details of a field, articles in professional journals might be, at least at first, a bit intimidating and difficult to understand.

In an attempt to fill this gap between popular and professional science literature, we decided to launch Reviews in Undergraduate Research (RUR), a journal which would solely publish review articles, written by undergraduates for undergraduates. Inside, you will find five articles ranging from molecular biology to space physics written by students across the United States. Also included is a special faculty review written by professors as an introduction for college students.

In opening this inaugural issue of RUR we would like to thank our generous sponsor Rice University and all of the people who helped contribute to this project.

Bahram Razani
Editor-in-Chief, RUR
Rice University, '03

Annual RUR

2002, Volume 1

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About the Cover:
T-47D cell nuclei stained with DAPI (blue) reveal two newly formed cells, products of a recent cell division, as their chromosomes begin to decondense in early telophase. See the article below by Will Renthal for a review of cell cycle checkpoints and the molecules involved in the decision making process.
(Photo: Courtesy of Bahram Razani and Dr. Rainer B. Lanz, Baylor College of Medicine)

Reviews in Undergraduate Research, Vol. 1, 1-7 , 2002

Cancer and the role of cell cycle checkpoints

Will Renthal[1], communicated by Dr. Eva Lee[1]

There exists an intricate network of proteins that continuously monitor each phase of the cell cycle to ensure proper replication. This network of proteins, termed checkpoints, first detects cellular abnormalities, and then coordinates their repair before the cell divides. The malfunction of these checkpoints often results in the proliferation of potentially damaged cells, and thus a tremendous susceptibility to cancer. This review will focus on the mechanisms by which checkpoints prevent the proliferation of damaged cells through each phase of the cell cycle, and how this understanding can provide novel targets for anticancer therapy.

The classic definition of cancer is "uncontrolled cell division." In a large, multi-cellular organism, uncontrolled cell division will soon result in large masses of rapidly growing cells (tumors), which cause significant damage to surrounding tissues. When tumors spread, they can damage vital organs and eventually cause death. In fact, cancer is currently the second leading cause of death in the United States, and thus cures for it would be of incalculable value. Current treatments of cancer involve exposing the patient to relatively nonspecific toxins, chemotherapy, in the hope that it will kill more cancer cells than normal cells. This type of medicine is a modern equivalent of 18th century bleeding treatments for bacterial infections. However, if clear biochemical differences between cancer cells and normal cell are discovered, chemotherapy could be improved considerably. Much as antibiotics only harm bacteria, novel anticancer drugs that only harm cancer cells can be developed through research. Since cancer is essentially the loss of cell division control, it seems prudent to search in these regulatory mechanisms for distinguishing characteristics of cancer cells. This review will present the general mechanisms which drive the cell cycle and what is currently known about the regulatory pathways that control it. It will then discuss how current anticancer therapies are taking advantage of cell cycle research.

The Cell Cycle

The Nobel Prize in Medicine or Physiology was recently awarded to three men, Leland Hartwell, Tim Hunt, and Paul Nurse, "for their discoveries of key regulators of the cell cycle" (www.nobel.se). Essentially every topic discussed in this review was in some way pioneered by these three men. The details they helped uncover may seem at first glance rather cumbersome, but it is these

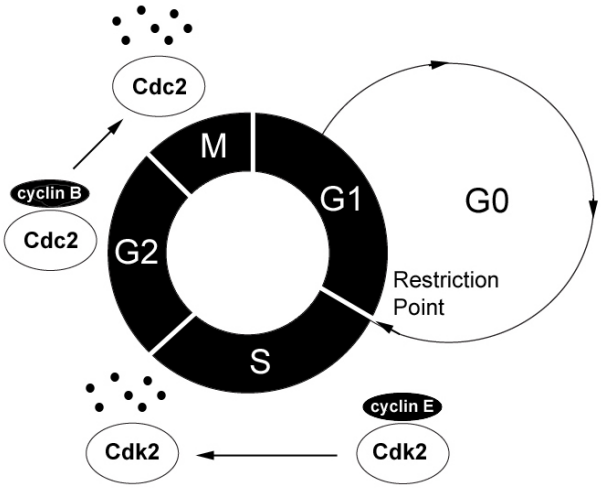

Figure 1. The Cell Cycle

The cell progresses through its division cycle, G1>S>G2>M, in the presence of growth signals and active CDK/cyclin complexes specific for each cell cycle stage. G0, a non-dividing but functional phase, occurs in the absence of growth signals. Finally, cyclin degradation is illustrated by the S-phase cyclin/Cdk complex (cyclin E/Cdk2) and the G2/M cyclin/Cdk complex (cyclin B/Cdc2), where each must be synthesized and degraded systematically for proper cell cycle progression into the next phase.

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very details that will eventually enable the development of targeted anticancer therapies.

Many of our cells can be triggered to divide upon the proper mitogenic stimulus (Cross and Dexter, 1991). A growth factor for example, can trigger a highly regulated unidirectional program that, when run successfully, results in the proper replication and division of the cell. This program, the cell cycle, must copy the parent cell's chromosomes and seal them in a safe daughter cell with all of the essential components needed for the daughter cell to function on its own. The human cell cycle accomplishes this in approximately 24 hours through four major phases: G1 (gap 1), S (DNA Synthesis), G2 (gap 2), and M (mitosis) (Lodish et al., 2000). Each phase serves a specific function to ensure proper cell division.

G1 – Gap 1

G1 takes about 9 hours to prepare the cell for DNA synthesis (S-phase) (Lodish et al., 2000). Though preparing the cell for S-phase may seem relatively uneventful, G1 is actually the most important regulatory phase in the cell cycle. It is in this phase that the cell decides whether to irreversibly continue the cell cycle through mitosis, or to enter G0, a quiescent phase during which the cell can function but not divide (Figure 1). This decision, called the restriction point in mammalian cells, is made in late G1 based predominantly on external growth signals (Lodish et al., 2000). Another key feature of late G1, which contributes to the unidirectionality of the cell cycle progression, is the priming of replication origins with MCM (minichromosome maintenance) proteins. The binding of these proteins to origins of replication is required for the initiation of DNA synthesis, but they can only bind to DNA in late G1 (Young and Tye, 1997). Thus, DNA synthesis is only initiated once – right at the G1/S transition.

S – DNA Synthesis

Once the cell has passed the restriction point, proteins synthesized in late G1 initiate the DNA replication machinery of S-phase. This delicate process of copying the parent cell's genome takes approximately 10 hours, and yields a single cell with two sets of each chromosome (sister chromatids) (Lodish et al., 2000).

G2 – Gap 2

G2 lasts about 4.5 hours and serves as a buffer to ensure the completion of DNA synthesis before the cell physically divides in mitosis (Lodish et al., 2000). Now with two sets of each chromosome, cell growth continues in order to double its size such that upon division two fully functional cells will result. A large amount of information is known about the G2/M transition, and it is discussed in detail below. This phase serves to synthesize proteins required for nuclear envelope breakdown, chromosome condensation, spindle formation, and other processes required for entry into mitosis (Maller et al., 1989). Many of these mitosis-promoting functions cannot be initiated until DNA synthesis is completed, thus serving as a buffer phase to prevent premature cell division.

M - Mitosis

Mitosis (nuclear division) is comprised of four substages during which specific events occur to separate daughter chromosomes from the parent's and enclose them in a new cell. Mitosis typically takes about 30 minutes in human cells, which is rather fast considering the complexity of this phase (Lodish et al., 2000). The first substage to occur is Prophase, in which the proteins synthesized in G2 break down the nuclear envelope of the parent cell, condense its chromosomes, and initiate spindle formation. Prometaphase follows as a transition period during which the sister chromatids shuffle until they align in the middle of the cell, which is then termed Metaphase. The sister chromatids then separate to opposite poles of the cell in Anaphase, which is followed its physical division in Telophase.

Cyclins and Cyclin-Dependent Kinases

The proper transition from each cell cycle phase to the next is dependent on two classes of proteins called cyclins and cyclin-dependent kinases. As the name suggests, cyclins are a class of proteins which are periodically synthesized and degraded, and the coordination of proper cyclin levels at the right time is essential for successful cell cycle progression. Cyclin-dependent kinases (Cdk) are a class of kinases whose catalytic activity is dependent on complexing with an appropriate cyclin. Once this complex is formed, the Cdk kinase activity is activated which results in the phosphorylation of many downstream effectors (reviewed in Udvardy, 1996). This phosphorylation serves to regulate the activity of the downstream effector, typically by activating or inhibiting it. One of the best studied cyclin/Cdk complexes involves cyclin B and Cdc2, also called MPF (Maturation Promoting Factor), and was first discovered by Yoshio Masui and Clement Market (Masui and Markert, 1971). It has been shown to serve many crucial roles in cell cycle progression such as nuclear envelope degradation and sister chromatid condensation in early mitosis (Maller et al., 1989) (Figure 1). These MPF-dependent processes are essential for the cell to efficiently divide the genetic information into daughter cells, and thus the activity of MPF is tightly regulated. If the cell allowed MPF to remain active through the latter stages of mitosis when the nuclear

envelopes are reforming, cell division would be prevented altogether. The way the cell deals with this problem is by ubiquitin-mediated proteolysis of cyclin B in late mitosis (Murray et al., 1989). Thus, the actively regulated levels of cyclin B mediate mitotic entry and exit. Without the synthesis of cyclin B prior to the G2/M transition, the kinase activity of Cdc2 remains inactive, and the cell can not enter mitosis. Without the subsequent degradation of cyclin B, the kinase activity of Cdc2 remains active and prevents the exit from mitosis.

Cell Cycle Control: Checkpoints

A cell cycle checkpoint is a general term used to describe a cellular process that stops or slows the cell cycle in conditions unfavorable for cell division (Hartwell and Weinert, 1989). This review will focus on the DNA damage cell cycle checkpoint. Since our cells undergo continuous bombardment by DNA damaging agents such as UV light and by-products of cellular metabolism, there exists an elaborate and evolutionarily conserved cellular DNA damage response that coordinates cell cycle progression with the repair of potentially mutagenic DNA damage (reviewed in Zhou and Elledge, 2000). Many of the proteins involved in the mammalian DNA damage response act as tumor suppressors and suggest that there are newly evolved repair and/or checkpoint genes critical in the maintenance of genome integrity, which highlights an additional importance of checkpoint control in mammals. The DNA damage response, like most cellular signaling pathways, involves first sensing a signal and then transducing it to downstream effectors that elicit the appropriate response. This review will present the recent studies, including some previously unreviewed, which have provided tremendous insight into many key components of this signal transduction pathway (outlined in Table 1).

Sensors

It is still unclear precisely how the cell senses damaged DNA, but a group of four fission yeast checkpoint proteins, Rad1, Rad9, Hus1, and Rad17, have been implicated along with their respective homologues in other organisms (reviewed in Lowndes and Murguia, 2000). It has been proposed that Rad1, Rad9, and Hus1 form a trimeric checkpoint-sliding clamp (CSC) similar in structure to the DNA polymerase clamp PCNA (Proliferating Cell Nuclear Antigen) (Venclovas and Thelen , 2000). By analogy to the loading of PCNA onto DNA by the RFC_{1-5} pentamer, the CSC is loaded onto DNA by $Rad17/RFC_{2-5}$, where Rad17 is a checkpoint protein that replaces RFC_1 in

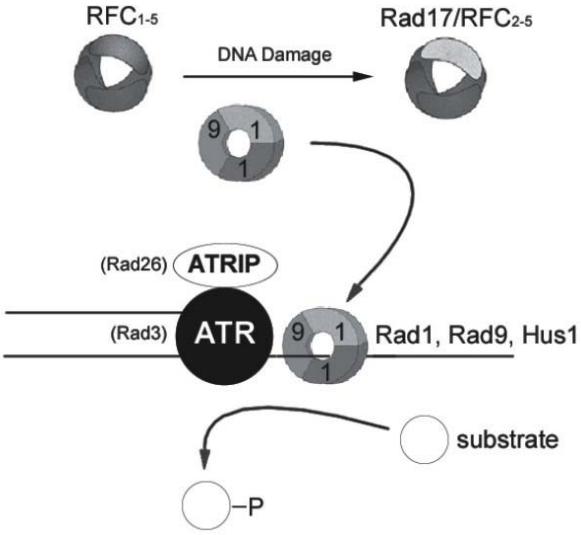

Figure 2. Substrate recruitment model for sensing DNA damage

Both ATRIP (Rad26) and the checkpoint sliding complex (CSC) Rad1, Rad9, Hus1, bind to the same sites of DNA damage. The CSC is loaded onto chromatin after DNA damage in a $Rad17/RFC_{2-5}$ catalyzed reaction. Once chromatin bound, the CSC recruits substrates of the ATR (Rad3) signal transducer kinase to propagate the DNA damage response.

the pentamer (Venclovas and Thelen , 2000). Once the CSC is loaded onto DNA, it could serve not only to recruit DNA polymerase but also to signal the activation of downstream DNA damage checkpoint proteins. In addition to the CSC and Rad17, the checkpoint protein Rad26 has also been implicated as a DNA damage sensor because it binds to and is phosphorylated by the Rad3 kinase independently of all other checkpoint proteins (Edwards et al., 1999). Thus, Rad26 has been proposed to be a sensor or at least far upstream in the DNA damage response. A recent model, the substrate recruitment model (Melo et al., 2001), provides some insight into the behavior of the putative DNA damage sensors Rad26/Rad3 and CSC/Rad17 in budding yeast (Figure 2). The model proposes that each complex is independently recruited to the same sites of DNA damage, and work in tandem for proper DNA damage checkpoint activation (Kondo et al., 2001; Melo et al., 2001). After the budding yeast CSC homologue is loaded onto DNA near sites of damage, it recruits various substrates of its Rad3 homologue to drive the signal transduction pathway of the DNA damage response. Though this model seems quite convincing in budding yeast, little is known about DNA damage sensing in mammalian cells (Melo et al., 2001). Recently, it was shown that the activation of both G1/S and G2 DNA damage checkpoints requires the phosphorylation of hRad17 by ATR (a Rad3 related kinase in mammals) and possibly ATM as well (Bao et al., 2001;

Post et al., 2001). However, unphosphorylated hRad17 can still load hRad9 of the CSC onto chromatin (Zou et al., 2002), so the significance of this phosphorylation remains unclear. Also, a recently cloned human protein, ATRIP, has some homology to the putative DNA damage sensor Rad26 (Cortez et al., 2001). ATRIP also seems to have many functional similarities to Rad26 including its tight association with and its phosphorylation by ATR (Rad3 related kinase), and a role in the G2/M checkpoint. Thus, human homologues of both of the putative DNA damage sensors in yeast seem to play similar roles in the human DNA damage response.

Transducers

Once DNA damage is sensed, the cell must transduce this signal down to its appropriate effector. In human cells, the activation of two kinases is essential for the proper transduction of DNA damage: ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad3 Related) (reviewed in Elledge, 1996; Zhou and Elledge, 2000). Little is known about precisely how ATM and ATR are activated, but a great deal has been uncovered about their respective roles in coordinating the DNA damage response. ATM was identified from a rare mutation found in the disease ataxia telangiectasia and leads to chromosomal instability and a high susceptibility to cancer (Savitsky et al., 1995). There are no known pathologies with ATR mutations, and ATR knockout mice die in early embryogenesis (Brown and Baltimore, 2000; de Klein et al., 2001). Upon activation, these similar kinases phosphorylate a number of target proteins, which transmit the DNA damage signal downstream, eventually arresting the cell cycle, initiating DNA repair, or, if necessary, causing cell death (apoptosis) (reviewed in Zhou and Elledge SJ, 2000) (Figure 3).

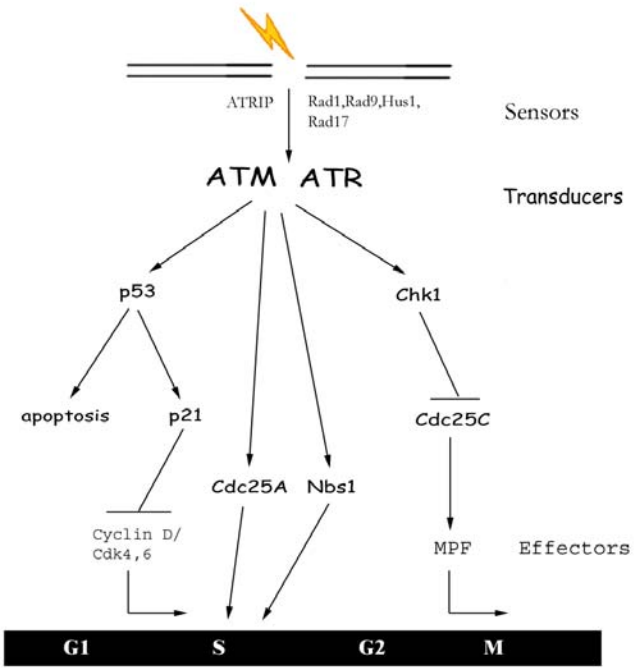

Figure 3. The DNA Damage Response

DNA damage is first detected by sensor proteins which in turn activate transducers in the signal cascade. These transducers then mediate the activation or inhibition of downstream effectors which can arrest the cell cycle or cause apoptosis.

Depending on when the DNA damage is sensed, ATM/ATR will activate a different axis of proteins. For G1 damage, ATM/ATR will phosphorylate p53, which then acts as a transcription factor for the synthesis of p21 (Cdk inhibitor) (Canman, et al., 1998; Banin et al., 1998; Tibbetts et al., 1999). Upon p21 expression, the G1/S transition is inhibited, preventing the synthesis of damaged DNA (Li et al., 1994). If damaged DNA is sensed during S-phase, the cell needs to slow DNA synthesis to provide time for repair. An ATM dependent pathway exists for an intra S-phase checkpoint in which ATM activation leads to the degradation Cdc25A (Falck et al., 2001). Since Cdc25A drives the initiation of DNA synthesis (Vigo et al., 1999), its degradation would allow synthesis to slow during S-phase and provide the necessary time for repair. This intra-S phase checkpoint is also controlled by the ATM phosphorylation of Nbs1 (Lim et al., 2000; Zhao et al., 2000; Wu et al., 2000), but the precise molecular mechanism remains elusive. Interestingly, Nbs1 is a protein involved in DNA repair as well, so this functional link between ATM and Nbs1 provides evidence of a high level of coordination between cell cycle progression and DNA repair. DNA damage incurred after S-phase results in the activation of the G2/M checkpoint to prevent entry into mitosis with damaged chromosomes. ATM/ATR also mediate this pathway by phosphorylating Chk1 in response to DNA damage (Chen et al., 1999; Zhao et al., 2001). This phosphorylation of Chk1 appears to enhance its kinase activity, which in turn phosphorylates Cdc25C (Sanchez et al., 1997). Cdc25C is inactivated by this phosphorylation and can no longer mediate entry into mitosis.

These signal transduction pathways (Figure 3) are just a few examples of the complex interacting network of proteins actually involved in processing DNA damage signals. The main idea embedded in these vast yet important details is that proteins (ATM/ATR) are activated upon DNA damage, and then trigger phase-specific cell cycle arrest and DNA repair.

Effectors

Name	Function
PCNA	Trimeric DNA clamp, holds pols on DNA
RFC_{1-5}	Pentameric complex, loads PCNA onto DNA
CSC – Checkpoint Sliding Clamp	DNA damage sensor? Structurally similar to PCNA
(Rad1, Rad9, Hus1)	
Rad17	DNA damage sensor? Complexes with RFC_{2-5} to load CSC onto DNA
Rad26 (fission yeast)	DNA damage sensor?
ATRIP (humans)	
Rad3 (fission yeast)	DNA damage transducer
ATR (humans)	
ATM	DNA damage transducer
p53	Transducer for G1/S checkpoint and apoptosis
p21	Cdk4,6 Inhibitor, involved in G1/S checkpoint
Cdc25A	Initiation of DNA synthesis, S-phase checkpoint
Nbs1	S-phase checkpoint transducer, DNA repair
Chk1	G2/M checkpoint transducer
Cdc25C	G2/M checkpoint transducer
MPF – Mitosis Promoting Factor	Necessary for G2/M transition
(Cyclin B/Cdc2)	DNA damage effector, G2/M checkpoint
Cyclin D/Cdk4,6	Necessary for G1/S transition
	DNA damage effector, G1/S checkpoint

Table 1. Components of the DNA damage response

Cell cycle checkpoint proteins and their respective functions. Putative, but not yet proven functions are followed by question marks.

The halting of the cell cycle is typically elicited by deactivating the Cyclin/Cdk complex involved in a specific phase transition (G1/S or G2/M). For example, p21 which is synthesized in response to DNA damage in G1, directly inhibits Cdk4,6 and thus prevents the transcription of proteins required for DNA synthesis. The final effector in the G2/M checkpoint is the CyclinB/Cdc2 complex (MPF) described earlier as being essential for the transition from G2 into mitosis. Upon DNA damage, the Cdc25C phosphatase can no longer remove inhibitory phosphates from Cdc2, and thus prevents the CyclinB/Cdc2 complex from breaking down the nuclear envelope, condensing chromosomes, and other events that occur in early mitosis.

Cell Cycle Controllers as Anticancer Drug Targets

Thus far this review has focused on the details underlying the control of cell division, but it is important not to lose sight of the exciting applications of this knowledge. For example, about half of all tumors have a damaged copy of the tumor-suppressor protein p53. Now that much of the detailed mechanism by which p53 inhibits tumor growth is understood, drugs can be developed to take advantage of its action. There are several biotechnology companies currently attempting to develop p53 therapies by reconstituting functional p53 back into tumor cells. This would restore the broken signal transduction pathway, and thus prevent tumorigenesis. The DNA damage checkpoint consists of many more pathways than those introduced in this review, and it is difficult to say at this phase which pathways will be of greatest utility in anticancer therapies. Thus, current cancer biology research is directed at better characterizing known pathways and elucidating novel ones involved in the DNA damage response.

About the Author

Will Renthal is currently a third-year Biochemistry Honors student at The University of Texas at Austin. There he conducts research as an Arnold and Mabel Beckman Scholar on the mechanism by which a novel antibiotic kills bacteria and as an NSF Fellow on MAP Kinase signal

transduction. For the previous two summers, he has researched cell cycle checkpoints with Dr. Eva Lee at the University of Texas Health Science Center at San Antonio, Institute of Biotechnology. His research focused specifically on characterizing the functions of a protein which is mutated in Nijmegen Breakage Syndrome (NBS) patients. This is a disease in which patients have an extremely high susceptibility to cancer and chromosomal instability. In his two summers of research, he has helped to clarify some of the subtle points about the NBS gene product, which is involved in both cell cycle checkpoints and DNA repair.

After his undergraduate education he plans to attend either an MD/PHD program or graduate school where he will further study the mechanisms underlying cell growth and development. Following this graduate training and a brief postdoctoral position, he aspires to become a professor at a medical center. There he hopes to conduct high quality basic research with a focus on drug discovery while teaching the next generation of scientists.

Acknowledgements

I would like to thank Dr. Eva Lee for providing me with two incredible summers of exciting research; Dr. Song Zhao for serving as my mentor and friend; Sean Post for stimulating discussions; and the rest of the lab for their support. I also thank Drs. Ann and Robert Renthal for their critical reading of this manuscript.

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Reviews in Undergraduate Research, Vol. 1, 8-20, 2002

The role of mechanical stress in skeletal myocytes: MAPK signal transduction pathways

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The idea of signal transduction provides the necessary link between a stimulus from the external environment of a cell, and the intricacies that occur among its intracellular components. The proteins embedded in the plasma membrane of a cell assist in this transduction in that they act as molecular antennae, capturing the initiating external stimulus and transmitting its effects to the cell cytoplasm. Albeit many molecules can freely pass through the cell membrane, those that are effectively excluded by this semipermeable barrier would have no way of communicating with the cell interior without the process of signal transduction. Consequently, many pivotal biochemical pathways would be completely hindered and the life of the organism affected would cease. The mitogen-activated protein kinase (MAPK) pathway is one of the most significant signaling systems used by an organism to elicit a variety of responses at the cellular level. This unique pathway is thought to be directly responsible for regulating cell proliferation, differentiation, and survival, all of which are vital for the existence of life. Even though the basic regulatory steps have been delineated, many fascinating features of this pathway are only beginning to emerge. The conventional study of signal amplification, which has historically encompassed the analysis of ligand interaction with cell membrane receptors, can be slightly modified to incorporate mechanical stress as the initiating component in the signaling pathway. Studying signal transduction in light of mechanical stretching may help enhance the understanding of pathway-initiating mechanisms in a way that can be applicable to mechanical manipulation of the extracellular matrix and plasma membrane domains. Such manipulation is seen *in vivo*, with normal physiological muscle contraction, as well as *in vitro*, with concentric and eccentric muscle stretching maneuvers. Furthermore, tracing the stimulus of cell stretching all the way to gene transcription demonstrates that many proteins from different families are involved in facilitating the activation of MAPKs. Theories on integrin signaling show how cell stretching induces the activation of protein kinase C (PKC), leading to the release of Ca^{2+} ions which are then sequestered by integrins. These active integrins may then activate focal adhesion kinases (FAK), which constitutes a major initiating pathway to activating MAPKs. Alternatively, integrins may immediately be activated by their associations with the extracellular matrix, which then activate FAK in a similar fashion. The dominance of ionic over mechanical activation is still unknown, and a combination of these mechanisms may contribute to the initiation of MAPKs. Observation of cell spreading and growth, two characteristics of activating MAPK pathways, can prove to verify the effects of mechanotransduction in muscle stretching. While the hypothetical models presented here provide a logical synthesis of concepts in signal transduction, the complexity surrounding the field leads to the idea that an intricate interplay of chemical processes within the world of the cell exists. The mediators of these processes include, but are certainly not limited to, integrin activation via calcium binding or dimerization of its subunits.

Key Words: Mitogen-activated protein kinase (MAPK), protein kinase C (PKC), integrin signaling, focal adhesion kinase (FAK), mechanotransduction

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A cell's decision to grow, proliferate, or terminate itself is the end product of long and complex deliberations. For instance, a quiescent (nongrowing) cell must receive and process a number of growth-stimulatory signals, notably those conveyed by growth factors, and assess whether its strength and number warrant entrance into an active proliferative phase. Decision-making such as this demands a complex signal-processing apparatus inside the cell. A helpful metaphor is an electronic circuit board constructed as a network of components that operate like resistors, transistors, and capacitors (Weinberg, 1998). Each of these components is a logical device that receives signals from other components, processes and interprets these signals, and then passes them on to other circuit elements. In the living cell, circuit components like these are proteins endowed with complex signal-processing capabilities. These proteins are capable of 'signal transduction' in that they receive signals, filter and amplify them, and then pass them on to other components. In this way, signal transduction allows the cell's external environment to govern intracellular machinery in an orderly fashion. Although there are many substances that can physically traverse the plasma membrane barrier, namely, hydrophobic substances and other relatively small molecules, there are still quite a few instrumental molecules that are effectively excluded by this barrier. Such substances would thus have no effect on cytoplasmic elements without the mechanism that is known as signal transduction. In this process, proteins function like molecular bucket brigades (Weinberg, 1998). A protein at the top of this brigade relays a signal to the next protein down the line, which in turn responds by transmitting the signal yet another step down. Such chains of command are commonly referred to as signal cascades. In actuality, the initiating signal is amplified virtually exponentially, in that each component that is activated in turn can activate several others to the extent that the effects are seen on a global scale. Of the known contributors to signaling cascades, mitogen activated protein kinases (MAPKs) are among the most versatile known. MAPKs are evolutionary conserved enzymatic complexes connecting cell membrane epitopes, or regions capable of provoking a cellular response, to regulatory intracellular endpoints. They respond to chemical and, as will be shown, mechanical stresses, in an effort to control cell survival and adaptation. MAPK activity is regulated through three-tiered cascades composed of a MAPK, a MAPK kinase (MEK), and an MEK kinase (MEKK) (English et al, 1999). These modules may be activated by small guanosine triphoshate (GTP) binding proteins, namely via G-protein linked receptors (Gutkind, 2000). In theory, all MAPK pathways activated in the forward direction, through substrate-level phosphorylation, can be inactivated by MAPK phosphatases.

Four parallel cascades of MAPKs have recently been described in mammalian cells, including extracellular signal-regulated kinase (ERK), stress-activated protein kinase p38, and cJun NH_2-terminal kinase (figure 1). An instrumental factor involved in the ERK cascade is Raf, which is a serine/threonine kinase that phosphorylates

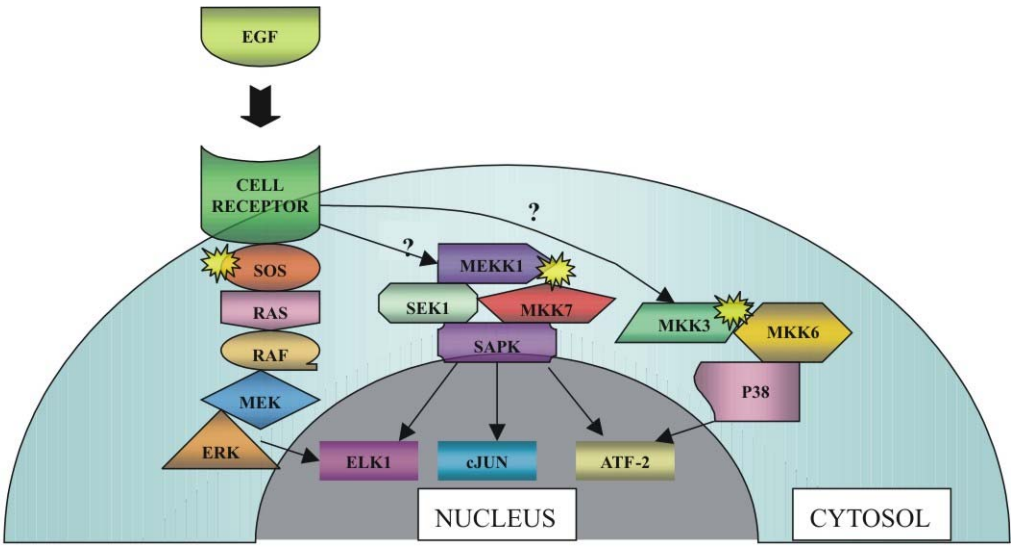

Figure 1. Hierarchy of MAPK signals

Signals from the cell surface are transduced through the cytoplasm by a cascade of protein kinases. In this case, epidermal growth factor (EGF) acts as a signaling promoter by binding to its transmembrane receptor domain with concomitant activation of Sos (the yellow sunburst symbol representing activation will be used throughout this article). The relationship between ligand binding and activation of both the SAPK and p38 pathways is still unclear. (Figure adapted from L.A. Tibbles, et. al.)

downstream targets in signaling pathways. MAPKs are activated via dual phosphorylation on threonine and tyrosine residues by MAPKs. Factors that have been shown to trigger MAPK activation include hormones, growth factors, reactive oxygen species, lowered pH, and mechanical stress (Widmann et al, 1999). Following activation, MAPKs can either phosphorylate different cytoplasmic targets or translocate to the nucleus and directly or indirectly affect transcription (Wretman et al, 2001).

The general pathway for stress activated protein kinases (SAPKs), which originates from members of the MAPK family, involves Raf, MEK, and ERK. The three main mediators of this pathway are ERK, SAPK, and p38, which each eventually have an effect on transcription factors in the nucleus (figure 1). As can be seen in figure 1, the ERK pathway is a hierarchical cascade originating at the cell membrane with receptors for either mitogens, which are substances that cause cells to undergo cell division, or growth factors. These receptors recruit, via adaptor proteins and exchange factors, the small guanosine triphosphatase (GTPase) known as Ras (Tibbles et al, 1999). Ras then subsequently activates Raf, a serine threonine kinase, which activates MEK (MAPK/ERK kinase). MEK, in turn, phosphorylates and activates ERK1 and ERK2 which translocate to the nucleus perhaps via a nuclear localization sequence (Alberts et al, 1994). These two proteins transactivate transcription factors, changing gene expression to promote cellular expansion, differentiation, or mitotic division.

It has been shown that signal transduction pathways are activated via stress and inflammatory mechanisms in mammalian somatic cells (Kyriakis et al, 2001). Environmental stresses such as physical exertion or mechanical manipulation of the muscle tissue constitute external stimuli that may employ MAPKs capable of initiating SAPKs (Tibbles et al, 2000). Both of these categories of stress will be discussed in turn, including their effects on the extracellular matrix and cytoskeletal agents.

As the regulation of mitogen activated cell signaling has been discussed in detail elsewhere (English et al, 1999), this article focuses on contemporary developments in understanding MAPK function in mammalian systems, coupled with stress-related stimuli from the external environment. The analysis of targeted mutations in mice and development of specific inhibitors have contributed to a greater understanding of the definitive role of MAPKs in mammals. It is becoming increasingly evident that MAPKs regulate almost all cellular processes, from transcription of genetic information to programmed cell death.

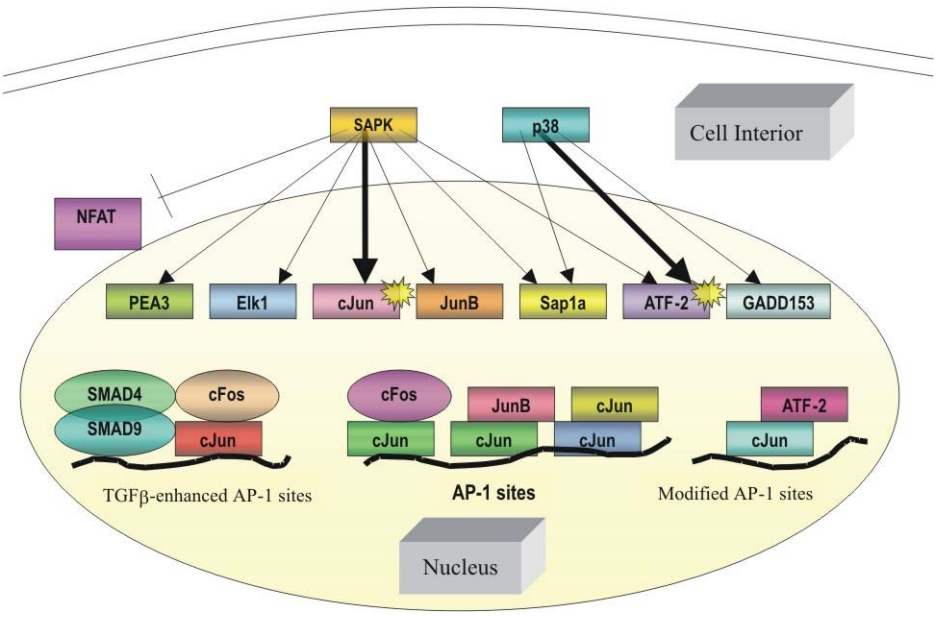

Figure 2. Transcription factor targets of the stress-response kinases

SAPK activation via an extracellular signal leads to the phosphorylation of specific downsteam transcription factors, leading to an effect at the level of the gene. Various forms of these transcription factors ultimately promote the transcription of genes with binding sites for the AP-1 complex. Arrows in bold correspond to major activation pathways outlined in Figure 1. (Figure adapted from L.A. Tibbles et al.)

Key components in MAPK activation

The stress activated protein kinases (SAPKs)

SAPKs reversibly bind and phosphorylate the transcription factor cJun (figure1). cJun is one portion of the activator protein 1 (AP-1) transcription factor complex; the remaining constituent parts include members of the cFos and cJun superfamilies. Transactivation of cJun (figure 2) by the SAPKs leads to increased expression of the genes that have AP-1 regions in their promoters (Tibbles et al, 1999). Among the primary targets of the AP-1 is the cJun gene itself, so transactivation of cJun initiates a positive feedback loop (Tibbles et al, 1999) which is presumably terminated upon transcription of the cJun gene. As can be seen in Figure 1, SAPK effectively acts as a universal pivot point, with targets to both a ternary complex transcription factor (ELK-1) and activating transcription factor 2 (ATF-2). The ternary complex factor ELK-1, once activated by SAPK, leads to positive regulation of the cFos promoter resulting in increased expression of the cFos protein with concomitant increases in AP-1 levels (Tibbles et al, 1999) (figure 2). Targeting of ATF-2, which can form heterodimers with cJun, is another suitable route to initiate increases in AP-1 expression. Given the myriad of possibilities for activating AP-1, it is quite apparent that the SAPK is a model transduction junction for amplifying a given extracellular signal. The SAPKs are encoded by at least three genes, and as with all MAPKs, each SAPK isoform contains a characteristic Thr-X-Tyr phosphoacceptor loop domain, where X indicates any amino acid structurally suitable for a loop domain (Kyriakis et al, 2001).

p38 protein kinases

p38 kinases respond to virtually the same agonists that activate the structurally similar SAPKs (i.e. members of the MAPK family), but under certain circumstances they are differentially regulated (Mendelson et al, 1996). As seen in figure 2, they activate via phosphorylating the transcription factor ATF-2 as well as growth arrest and DNA damage transcription factors (Tibbles et al, 1999). As will be discussed later, p38 activation can be mediated by protein kinase C (PKC) by an experimentally unidentified mechanism (Ryder et al, 2000). Muscle contraction has been implicated in activation of PKC in response to electrical stimulation, however (Richter et al, 1988). As a checkpoint prior to nuclear translocation, p38 appears to be instrumental in regulating a variety of cellular processes, ranging from maintenance of genetic information to preservation of the cell line.

ERK- a third class of Stress-Activated MAP Kinases

The extracellular regulated protein kinases (ERK), with a phosphoacceptor sequence of Thr-Glu-Tyr, contain an NH_2-terminal kinase domain followed by an extensive COOH-terminal tail of unknown function that has several proline-rich motifs indicative of binding sites with SH3 domains (Zhou et al, 1995). These SH3 adaptor proteins are instrumental in linking the initial activation of a kinase to the downstream components of any signal transduction pathway. Although the stimuli that recruit ERK kinases have not been well identified, environmental stresses such as osmotic shock and oxidant stress have been shown to substantially activate ERK and similar substrates (Abe et al, 1996). EGF activation of ERK has been subsequently documented in studies done on cultured cells (Chao et al, 1999) suggesting that a MAPK may be involved (figure 1).

Effects of myocyte stretching on MAPKs

As previously mentioned, there are a variety of environmental stimuli that activate MAPK pathways. Of these stimuli, manipulation of the cell plasma membrane and associated extracellular matrix appears to be especially effective in propagating signal transduction pathways. Voluntary stretching of muscle tissue, exercise induced muscle contraction, and inflammation of muscle cell (myocyte) tissue are all examples of such manipulation of the cell exterior. While many hypotheses and models for mechanotransduction exist, the role of tensile and shear forces on activating MAPKs will be the focus here.

Concentric and Eccentric contractions

Studies on isolated rat skeletal muscle have shown an increase in phosphorylation of both ERK and p38 MAPKs via mechanical alterations, whereas an increase only in ERK activity was caused by contraction-related metabolic/ ionic changes (Wretman et al, 2001). The latter effectors for ERK activation stem from acidotic cell conditions, in which the buildup of lactic acid from cellular respiration induces, either directly or indirectly, the activation of MAPKs. This idea will have some interesting implications in exercise-induced stimulation of MAPKs, as will be discussed in the following section.

Concentric muscle contractions (i.e. contractions induced in muscle fibers along a common axis situated at the geometric center) have been found to have divergent effects on MAPKs in that they induce a marked elevation in ERK phosphorylation, whereas p38 is not significantly affected (Wretman et al, 2001). In addition, the increase in phosphorylation of ERK, but not p38, can be induced by metabolic changes, such as acidification, that occur during

repeated contractions and also by mild mechanical perturbations (Wretman et al, 2001). Eccentric contractions (i.e. contractions induced in muscle fibers along an axis other than that situated at the geometric center), on the other hand, seem to markedly facilitate the phosphorylation of both ERK and p38 MAPKs (Wretman et al, 2001). Thus there seems to be an alternative mechanism at work in concentric contractions that selectively stimulates the initiation of ERK and not p38. It may be hypothesized that the concentric contractions do not generate sufficient force to exert an effect on the p38 pathway, which may give rise to the idea that the upstream components of the p38 activation pathway are more internal to the cell surface, in relation to the ERK elements. However, there still remains much to be discovered about the nature of these concentric/eccentric contractions and their relationship to the activation of these MAPK components.

The fact that p38 MAPK phosphorylation is not affected greatly by concentric contractions implies that it is little affected by metabolic alterations, and previous studies have shown no effect on phosphorylation by acidotic conditions (Wretman et al, 2001). Furthermore, since Wretman has shown that p38 phosphorylation is not induced by mild mechanical stress, by exclusion the higher mechanical stress imposed upon muscle in isometric contractions is required to induce an increase in p38 MAPK phosphorylation. This was verified in Wretman's studies by the finding that eccentric contractions markedly induced phosphorylation of p38 MAPK, an effect that also tended to occur with severe stretching maneuvers. The importance of mechanical stress in enhancing MAPK phosphorylation is now becoming increasingly evident. As far as the mechanism underlying MAPK phosphorylation with eccentric contractions is concerned, it appears that a number of internal cytoskeletal elements are involved. In eccentric contractions, the contractile units (cross bridges) and the elastic elements in series (z-lines and tendons) are involved in force generation and transmission, whereas in severe stretch, stiffness of elastic components in parallel (sarcolemma, endomysium, perimysium, and epimysium) and in series generates force (Wretman et al, 2001).

Exercise stimulation

It has been shown that exercise- and contraction-induced ERK signaling involves the same Ras/Raf/MEK pathway in the activation of ELK1 (Aronson et al, 1997) (figure 1). PKC activation is also known to lead to Ras activation and thus stimulate MAPK activity (van Biesen et al, 1996). Additionally, PKC can mediate p38 MAPK activation by an unidentified mechanism. One possible mechanism may involve a signal cascade, in which PKC phosphorylates a MAPK at its tyrosine and threonine phosphoacceptor domains (figure 3). The activation of PKC occurs via a G-protein, which is itself activated by the binding of an agonist ligand to its specific cell receptor (figure 3). The mediator, phospholipase C, cleaves phosphatidylinositol bisphosphate (PIP) into diacylglycerol (DAG) and inositol triphosphate (IP), the former of which directly activates PKC (Voet et al, 1995).

As previously mentioned, the activation of PKC has been

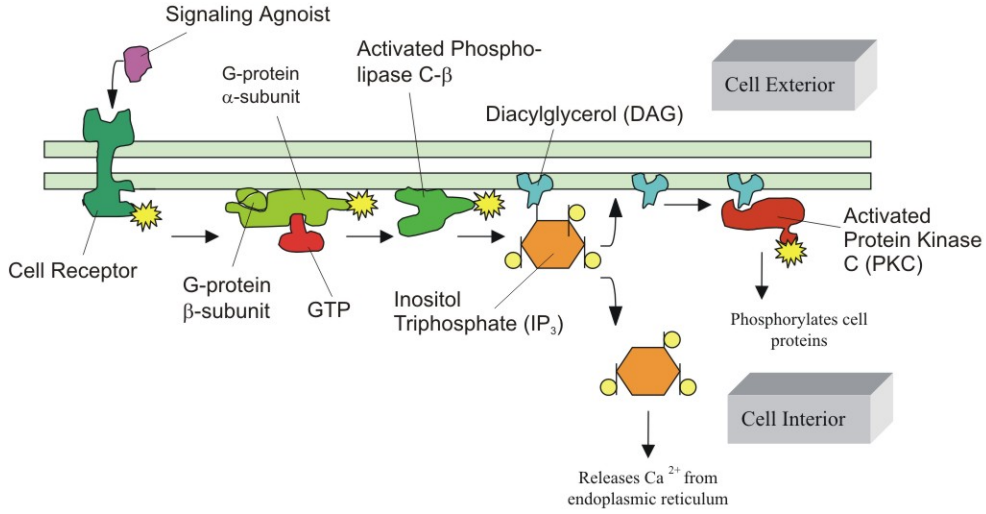

Figure 3. Activation of Protein Kinase C (PKC) via G-proteins

The activation of PKC is preceded by a number of steps, originating from the binding of an extracellular ligand that activates a G-protein on the cytosolic side of the plasma membrane. The G-protein, using GTP as an energy source, then activates PKC via the phosphatidylinositol bisphosphate (PIP2) intermediate, which is shown as the DAG/IP3 complex. (Figure adapted from Alberts, B. et al)

suggested to be caused by muscle contraction in response to electrical stimulation (Cleland et al, 1989). Coupling this idea with myocyte stretching might provide a relationship between the presence of an external force and the binding of the activating ligand. If one would imagine this ligand in the vicinity of the extracellular matrix, the direction and magnitude of force in cell stretching would become a major determining factor in the orientation and proper binding of the agonist to its transmembrane receptor. This would support the selective activation of p38 MAPKs only in eccentric contractions. Moreover, the selective responsiveness of certain upstream elements of MAPKs (i.e. PKC) to electrical stimulation may be the result of ionic changes within the cell *in vivo*.

Energy considerations within a cell and acidification of the cytosolic environment can also shed light on the regulation of MAPKs. When energy turnover in contracting muscle is high, the intracellular milieu becomes acidic due to the buildup of lactic acid discussed earlier (Aronson et al, 2000). A high energy turnover is indicative of a high rate of cellular respiration, which in turn is the result of active muscle contraction during physical exertion. *In vitro* analysis of muscle stretching indicates that acidosis, of the magnitude similar to acidosis in severe fatigue, can induce the ERK MAPK phosphorylation in skeletal muscle cells (Fitts, 1994). Thus the analogous process of *in vivo* myocyte stretching from physical exertion can be seen in myocyte stretching *in vitro*.

Ca^{2+} and positive feedback hypothesis

The aforementioned response of MAPK to electrical stimulation is noticeable in fluctuations of established electrochemical gradients within living cells. Perhaps one of the most salient ions in myocytes is Ca^{2+}. The presence of calcium ions allow for the operation of the contractile machinery within myocytes of all types. Via a well-established model, calcium ions bind to the tropomyosin protein on actin filaments, and subsequently expose various binding sites for myosin. In this way, the myosin head can bind to the actin filaments and allow for muscle contraction to occur. The presence of calcium ions may be correlated to the presence of mechanical stress on the exterior of the cell since the influx of ions through stretch sensitive mechanoreceptors has been implicated in inducing muscle contraction *in vitro* (Aronson et al, 2000). Once in the cytosol, Ca^{2+} ions exert an effect on a variety of cellular elements. Figure 4 illustrates the indirect role of PKC in releasing calcium ions. One of the major products of PKC activity, IP_3, is responsible for activating calcium voltage-gated channels in the membrane of the endoplasmic reticulum. This *in vivo* process mimics the activation of mechanoreceptors seen in many *in vitro* studies (Aronson et al, 2000). Thus, there appears to be a relationship building between the role of mechanical cell membrane stretching and calcium signaling. An intricate network of related pathways seems to be developing, since it was previously mentioned that PKC activates ERK and p38 pathways. It is possible to imagine a signaling pathway propagated by calcium ions that is directly responsible for activating these MAPK pathways. Such calcium signaling could conceivably originate in a way similar to the model in figure 5. Such a PKC-mediated pathway, however, has not yet been experimentally described.

A major consideration not yet addressed is the regulatory mechanism of this postulated signaling system. In normal physiologic systems, the effects of calcium are quickly dissipated unless there is a positive feedback mechanism to fuel the continuation of the calcium-triggered process in question. The initial release of Ca^{2+} ions may prompt an even greater release of calcium into the cytosol, as some ions can bind to allosteric sites on voltage-gated calcium channels, inducing release from reservoirs such as the sarcoplasmic reticulum in myocytes (Alberts et al, 1994). Such a feedback mechanism is supported in evidence of studies on smooth muscle. These studies illustrated that an initial calcium release induces an even greater output of systemic calcium to produce an extended

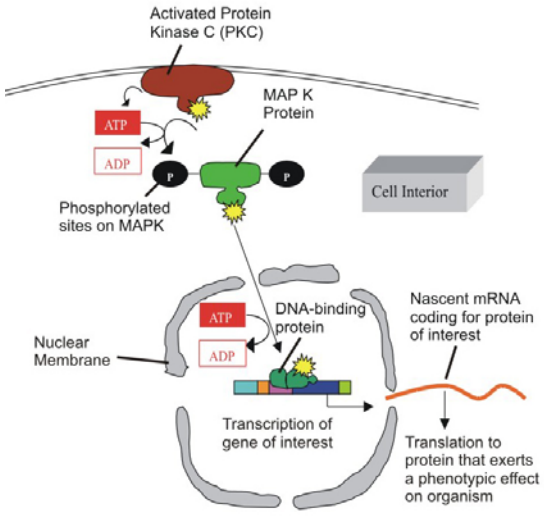

Figure 4. Intracellular pathway of MAPK activation via PKC

Activated PKC phosphorylates MAPK on its tyrosine and threonine sites, at the expense of two ATPs. MAPK then phosphorylates its downstream targets (not shown) to the level of a transcription factor that binds to a DNA element and prompts the transcription of mRNA coding for the protein of interest. Such proteins are employed in cell differentiation, proliferation, and even death.

(Figure adapted from Alberts B. et al)

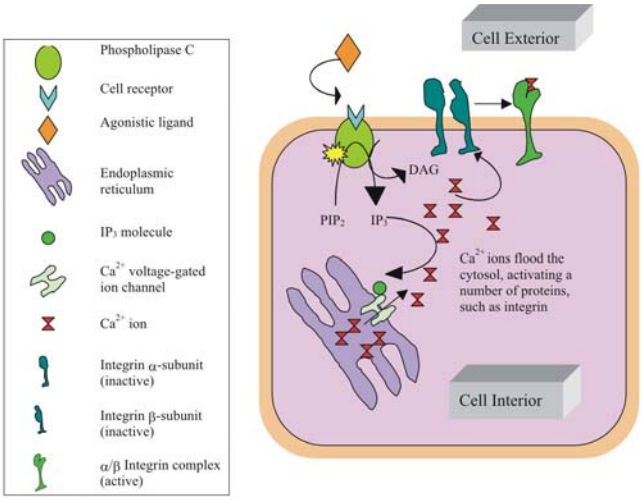

Figure 5. Role of IPs in Ca2+ release

IP3 formation during PKC activation has some interesting effects within the cytosol. A well-characterized effect of IP3 is binding to Ca2+ voltage-gated channels, inducing a conformational change that allows calcium ions to flow down their electrochemical gradient (from the lumen of the endoplasmic reticulum toward the cell interior). The presence of calcium ions within the cytosol allows them to interact with a number of calcium dependent proteins, such as integrins. Interestingly enough, their binding to such proteins effectively removes them from the cytosol, thus increasing the demand for free Ca2+ in the cell interior and stimulating the release of even more Ca2+ ions. This positive feedback loop will be important in recruiting multiple myocytes at the tissue level. (Figure adapted from Voet D., et al)

effect, such as prolonged intestinal contraction within the digestive system (Katoch et al, 1999). Extended contraction is not normally observed in skeletal muscle, except under tetanic conditions in which multiple simple muscle spasms are combined into an apparently smooth continuous effort. Even so, this positive feedback control of calcium can be involved in systems that sequester calcium for purposes of signal transduction, as will be discussed next.

Integrin signaling: a special case

Integrins comprise a major family of transmembrane proteins that allow for both cell-cell and cell-matrix associations. Although most integrins are cell-matrix, those that are involved in cell-cell contacts bind heterophilically to extracellular matrix elements on adjacent cells. Integrins are also found in hemidesmosomes (major cell surface attachment sites at contacts between the cell membrane and components of the extracellular matrix), where they connect to intermediate filaments inside the cell, as well as in focal adhesions (cell-matrix adherens junctions) where they connect to actin filaments and stress fibers (Alberts et al, 1994). The latter form of integrin interactions proves to be noteworthy in activating MAPK pathways in response to cell stretching.

There are four major characteristics of integrin that make it particularly unique as a signaling molecule (Alberts et al, 1994): (1) multiple integrins each recognize different targets (fibronectins, laminins, etc), (2) integrins can be regulated (e.g. during mitosis, phosphorylation of the cytoplasmic tail of the b-subunit of integrin impairs its ability to bind fibronectin, an extracellular matrix protein involved in cell adhesion and migration, causing the cells to round up), (3) matrix binding to integrin regulates cellular activities through focal adhesion kinase (FAK) (figure 8) signaling cascades, and (4) integrins can be either Mg^{2+} or Ca^{2+} dependent. Each of these four attributes can contribute to a greater understanding of integrin function at the level of signal transduction.

Mechanical activation

There are two main subunits for integrin proteins: the a- and the b-subunit. While there are many binding sites for a variety of proteins on each of these subunits, the focus will be on the binding site for laminin, located on the a-subunit, and the binding site for fibronectin located on the b-subunit (Disatnik et al, 1999). The intracellular signaling cascades that are activated when integrins bind to their extracellular ligands are varied. Biochemical changes in cells with integrin deficiencies indicate that integrins are true signaling molecules, transmitting information from the extracellular compartment into the cell in what constitutes “outside-in signaling” (Hynes, 1992). The current fluid-mosaic model of transmembrane proteins in the plasma membrane and the associated extracellular matrix holds that the cell membrane components are not static but rather in constant motion (Alberts et al, 1994). It is not outlandish to consider a force from mechanical stretching of this fluid membrane that is sufficient in magnitude to bring together certain transmembrane proteins of interest. This is in fact what happens with integrins during membrane stretching (Disatnik et al, 1999). One of the earliest changes initiated by integrin engagement is clustering of integrins at focal adhesions and tyrosine phosphorylation of proteins such as paxillin (a cytoskeletal component that localizes to the focal adhesions at the ends of actin stress fibers), talin (a cytoplasmic protein that links integrins to the actin cytoskeleton), and FAK (a cytosolic tyrosine kinase which is recruited at an early stage to focal adhesions and mediates many downstream cellular

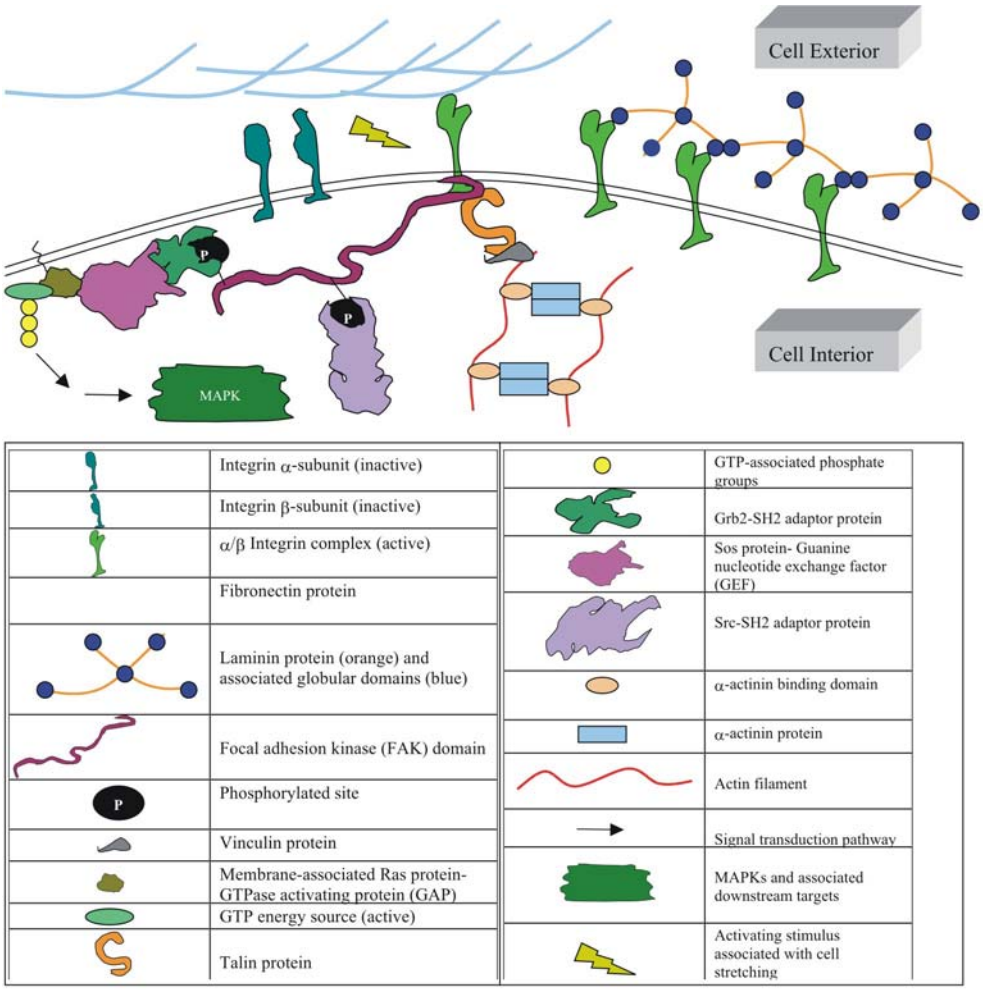

Figure 6. Model of MAPK activation via proteins of the extracellular matrix and cytoskeleton

Stretching the plasma membrane may cause a number of changes in the surrounding extracellular matrix and internal cytoskeletal architecture. These changes may, in turn, activate a MAPK signal transduction pathway, leading to a number of effects manifested in cell growth, differentiation, and proliferation. Two mechanisms may be at work here. First, tension in the cell membrane may prompt an association of a and b integrin subunits from Ca2+ binding, thus activating them. These associated units may then further activate focal adhesion kinase (FAK), with eventual activation of MAPK. Another possible mechanism may involve the association of actin filaments to the vinculin linker protein, which may activate FAK in a similar fashion. The latter mechanism may also involve the binding of Ca2+, perhaps in preparation of muscle contraction in active myocytes. In reality, a combination of these two processes may occur. (Figure adapted from Cooper, et al)

responses) (Schaller et al, 1992). FAK phosphorylation is considered to be one of the critical steps in the downstream signaling that promotes cell spreading and cell survival (Disatnik et al, 1999). Although the details of these more distal events remain to be elucidated, there is evidence that the binding of integrins to their extracellular ligands may activate pathways that prevent apoptosis in a variety of cell types, including skeletal myocytes (Zhang et al, 1995). Association of integrins with concomitant binding of fibronectin to the b-subunit is sufficient to form an activated dimer of integrin subunits. Close inspection of figure 6 illustrates that this aggregation of integrin leads to the association of various proteins, including FAK and talin, as well as their localization to focal adhesions (Kornberg et al, 1992).

It has been experimentally shown that the activation of PKC is necessary for the interaction of b integrin with fibronectin to promote FAK phosphorylation and spreading of muscle cells (Disatnik et al, 1999). Woods and Couchman found that activation of PKC leads to the localization of proteins such as talin to focal adhesions. Using specific PKC inhibitors, Haimovich *et al*. showed that PKC plays a crucial role in integrin signaling and phosphorylation of FAK in platelets. It has also been

shown that PKC isoforms translocate to nuclear structures and focal adhesions upon binding of vascular smooth muscle cells to fibronectin (Vuori et al, 1993). This adds to the growing evidence of the importance of PKC in both integrin signaling and MAPK activation, which can be mediated by activated FAK. Since it is known that MAPKs allow for growth and differentiation of cells (English et al, 1999), the observable changes in cell spreading induced by associations of integrins, which indicate growth patterns, must be the result of MAPK signal transduction.

Ionic activation

Integrin subunits also contain binding site for Ca^{2+} and Mg^{2+} on both the a- and b-subunits (Alberts et al, 1994). As noted earlier, one of the characteristics of integrins is that they are highly regulated, often by means of these divalent cation binding sites. Integrins can be activated by Ca^{2+} binding to its appropriate receptor on the transmembrane protein. Recalling the previously discussed considerations of calcium ions in cell stimulus response, it is clear that integrin has an important role in responding to mechanical stimuli. The interrelationship of PKC, Ca^{2+} ions, and integrins is beginning to be revealed: cell stretching can induce the aggregation of integrins, thus activating them. These activated integrins may then stimulate the initiation of a signal cascade through MAPK, in a postulated mechanism delineated in figure 6. The previously discussed Ca^{2+} positive feedback mechanism mediated by IP_3 binding to the endoplasmic reticulum (figure 3) may constitute the fuel needed to keep the cycle of MAPK activation running by allowing more calcium to bind to the integrin divalent cation sites. Furthermore, the activation of PKC is made possible by the presence of IP_3 (figure 3). By syllogism, this is how the action of active PKC is involved in integrin signaling. This somewhat refines the rather crude model of the positive feedback mechanism described earlier, in which Ca^{2+} binds to other voltage gated ion channels, inducing the release of more calcium ions.

Recruitment of myocytes in tissue systems

The aforementioned considerations have thus far pertained to a single cell. While it is important to understand the mechanics of cellular processes, it is equally important to investigate what occurs with multiple cells at the tissue and organismal level. Taking this into

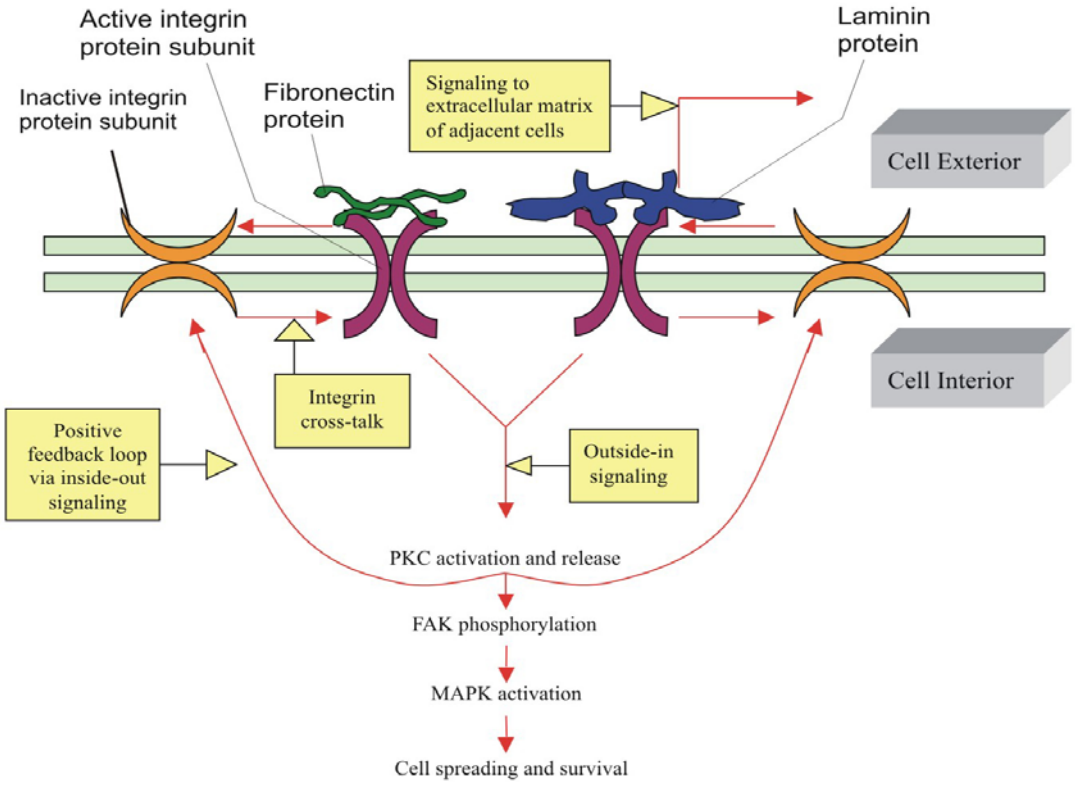

Figure 7. Activation of integins via positive feedback sources: outside-in and inside-out signaling

Clustering of integrins due to an external stimulus can induce them to become activated, with subsequent activation of PKC and downstream phosphorylation of MAPKs. These active integrins can then activate other neighboring inactive integrins in what constitutes "integrin cross-talk" via inside-out signaling. This inside-out signaling proves to be a form of positive feedback, in which more integrins are recruited to allow for cell spreading and growth, a phenomenon regulated by MAPKs. Note that since phosphorylation of FAK is the result of integrin activation, integrins are partially responsible for initiation of MAPK signal transduction pathways. (Figure adapted from Disatnik, et al.)

consideration calls to mind a mechanism of signal spreading, in which a few cells propagate an activated signal pathway to adjacent, or nearby cells. Understanding such a mechanism proves to be essential, since many biochemical processes hardly involve only a single, isolated cell. Moreover, physiology and pathology are meaningless outside the context of cell aggregates.

Although integrin engagement leads to signal cascade activation, it is also clear that the process of muscle cell attachment and spreading involves an activation of integrins themselves, which then allows them to execute "inside-out signaling" (Disatnik et al, 1999). In this form of signaling, integrin has an increased affinity for its extracellular matrix ligand, such as laminin (figures 6 and 7). This activation of integrins by binding laminin promotes the cell adhesion that may be an important step in the morphological changes that cells undergo when spreading on a solid substrate (Disatnik et al, 1999). It turns out that PKC activation is sufficient to promote inside-out signaling and since it has already been shown that PKC is necessary for this signaling, a positive feedback loop is created (Disatnik et al, 1999). Indeed, the gradual morphological changes associated with cell spreading suggest a multistep process involving first the detection of the extracellular environment by the cell and then a progressive change of the cell membrane to interact with that environment (Disatnik et al, 1999). This is demonstrated most clearly by the fact that the changes do not occur when cells are plated in the absence of immobilized matrix proteins to which integrins can bind (Chen et al, 1994). The presence of such proteins initiates a signaling cascade inside the cells, and the cells in turn both alter their membrane properties to interact with the ligands and organize these ligands into a complex matrix (figure 6). A positive feedback loop is intrinsic to such a process (Disatnik et al, 1999) and this further refines the initial feedback model described earlier, thus completing the understanding of its significance in signal transduction.

The positive feedback loop of integrin engagement, signaling, and activation is shown in figure 7. Integrins propagate their activated signal to other cells, via a dynamic equilibrium between an active state and an inactive state (Disatnik et al, 1999). When there is a sufficient number of active integrins for effective engagement with their extracellular ligands, outside-in signaling is initiated, leading to an increase in PKC activity, a further increase in integrin activation and affinity (via inside-out signaling), and further outside-in signaling (Disatnik et al, 1999). This positive feedback loop promotes biochemical changes, including FAK phosphorylation and focal adhesion formation as seen in figure 6, which in turn lead to a downstream cascade of biochemical changes leading to gene expression as shown in figure 4. Inside-out signaling may also be effective in transducing a signal of interest to nearby or adjacent cells. The affinity of integrin to laminin, an extracellular matrix protein involved in cell to cell interactions, may allow for this to occur. As specific inactive integrins are recruited to become active, via the proposed positive-feedback mechanism, they may bind laminins and change conformation. This binding may cause a widespread agitation in the external environment in a cell, altering the orientation of the extracellular matrix surrounding neighboring cells. The fibronectin in these nearby cells may in turn be brought into closer proximity to the plasma membrane, thus facilitating the activation of transmembrane proteins, such as integrins, in the same way as that observed for the initial cell. By this process, integrins from completely different cells can "talk" to each other in a rather elaborate communication scheme, allowing for a more global response to the initiation of a MAPK signal transduction pathway.

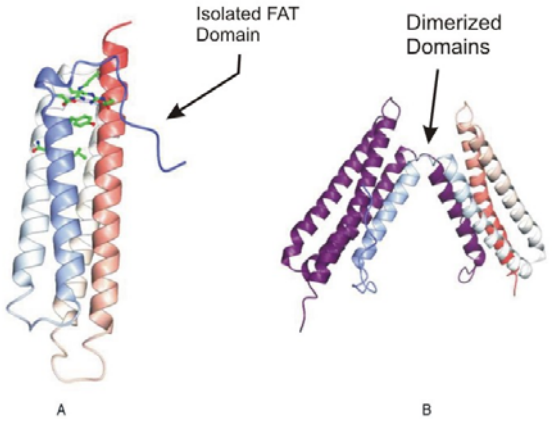

Figure 8. Two structures of the focal adhesion targeting domain of focal adhesion kinase

The localization of focal adhesion kinase (FAK) to sites of integrin clustering initiates downstream signaling. The C-terminal focal adhesion targeting (FAT) domain causes this localization by interacting with talin and paxillin (not shown). Isolated FAT folds into a four- helical bundle (A), which has the capacity to form domain-exchanged dimers in which the N-terminal (B). A structure-based alignment including these proteins and the vinculin tail domain reveals a conserved region which could play a role in focal adhesion targeting (Figure courtesy of Noble, et al).

Conclusion

MAPKs have been shown to be relatively ubiquitious in their activity, yet their activation is quite specific. This review article has introduced MAPKs in the conventional

light of signal transduction activation: the binding of a free ligand (i.e. EGF) to its specific receptor, and the subsequent downstream effects associated with such activation. To provide a more provocative contrast, the idea of mechanical stretching was introduced along with its role as a signal transducer. Albeit the binding of tangible elements from the extracellular matrix is involved in mechanotransduction, the overall induction of mechanically activated signal transduction pathways are initially employed by the intangible presence of tension and mechanical stress in the cell plasma membrane and associated proteins.

To further supplement this unconventional perspective on signal transduction, the hypothetical model of a positive feedback mechanism was discussed, involving the employment of calcium ions that originated from the activation of PKC and other cytosolic components. The final aspect that drove home the involvement of MAPKs in this feedback mechanism was the observation of cell spreading and cell growth, two events that are hallmarks of MAPK activation. While many of the mechanisms and processes discussed in this review are purely hypothetical, and should be treated as such, they are the products of the logical synthesis of concepts in MAPK signal transduction. Thus, in reality, a combination of events such as calcium activation, rearrangement of extracellular proteins, activation of transmembrane domains, and transcription at the level of the gene may all be occurring. Furthermore, these processes may be the result of other components not discussed in this review. Nonetheless, the models presented in this article do provide an integrative and innovative approach to signal transduction that may help in future discoveries.

Prospects

As is the case with most cell analysis, genetic models are always indispensable in the dissection of various signal transduction pathways. Although there has been a multitude of such genetic models from which to draw conclusions as to stress-activated MAPK pathway regulation, new emerging genetic models such as the dorsal closure pathway in *Drosophila*, coupled with the completion of the *C. elegans* and other genome sequencing projects, should make it possible to understand the epistatic relationships between MAPKs and their upstream activators (Tibbles et al, 1999).

Understanding these pathways in the context of human physiology and disease pathology is much more of a challenge. With regard to skeletal myocytes in particular, further study of knockout and transgenic mice may provide the link between signal transduction and muscular dystrophy, for example. A deficiency of the molecule a7 integrin has been positively linked to various cases of congenital muscular dystrophy. Muscular dystrophy, which causes a progressive deterioration of the muscle fiber architecture, seems to be slightly correlated to the insufficient presence of integrin. Since it is known that integrin helps establish the complex organization of the cell membrane, a lack of it must disrupt the integrity of the membrane leading to the onset of the genetic disease. Thus, a more fundamental understanding of integrin and related transmembrane proteins may help assist in elucidating possible treatment, or perhaps even a cure, for muscular dystrophy. However, paramount discoveries in the exact pathways of such debilitating muscle disorders can only be realized with a more aggressive, genetic approach to understanding signal transduction at its most fundamental core: the activation and initiation of a signaling pathway. Once this can be fully divulged for a particular skeletal muscle disease, then perhaps an inhibitory genetic mechanism can be proposed, in which symptoms can be fully repressed before they are manifested phenotypically.

At the current state of genetic engineering, the presence of specific genes in humans cannot be controlled. While this may at first seem like a major setback for the millions of people afflicted with skeletal muscle diseases and other genetic ailments alike, there appears to be a great deal of hope in fighting these diseases using knowledge of signal transduction. This is to say that while the transmission of a gene from one generation to another is difficult to control, the first major point of intervention may be at the level of transcription and translation of a gene, which is controlled by signal transduction. Gene therapy currently seems to be a promising solution for treatment of various genetic diseases. Gene therapy can be targeted to somatic (body) or germ (egg and sperm) cells. In somatic gene therapy the recipient's genome is changed, but the change is not passed along to the next generation. In germline gene therapy, the parents' egg and sperm cells are changed with the goal of passing on the changes to their offspring. Germline gene therapy is not being actively investigated, at least in larger animals and humans, although a lot of discussion is being conducted about its value and desirability. And while complete obliteration of genetic disease does not seem to be in the near future, regulating these genetic diseases before they get out of control is definitely within reach as more research is done on signal transduction.

About the Author

David A. Barron grew up in El Paso, TX, where he developed an early affinity for biomedical research through voluntary experiences in the local area medical center. He attends Rice University, where he is working towards completion of a B.A. in Biochemistry and Cell Biology. Although the majority of his coursework is completed at Rice, he has studied at Oxford University in Oxford, England and through the Baylor College of Medicine, in the Texas Medical Center, both institutions of which have enhanced his knowledge of the biosciences tremendously. He is currently engaged in a research project for the Department of Surgery at Baylor, examining the mechanical and structural properties of both desmin- and integrin-deficient mice as seen in biochemical assays and tissue stretching maneuvers. Past findings have led him into the realm of signal transduction, specifically investigating the influence of mechanical forces on the extracellular matrix. The research team he works with, led by Aladin Boriek, Ph.D., hopes to find a specific role of such mechanical signaling at the tissue level, with the long term prospect of developing a further understanding of debilitating muscular diseases resulting from genetic disorders.

Mr. Barron plans to continue his research during his final year at Rice, with the possibility of undertaking a joint Rice/Baylor research project in an attempt reach a synthesis of different ideas in his particular field of study. While still considering the possibility of pursuing an M.D./PhD. program, his career goals are primarily oriented towards earning an M.D. degree in order to apply his knowledge of biochemistry to patients in a health care setting. In either case, he plans to actively partake in research projects tailored toward dealing with human disease at both the macroscopic and microscopic level, so that he may ultimately examine disease states and pathologic patterns from both a scientific and clinical perspective.

Acknowledgements

Special thanks to Dr. Aladin Boriek and Dr. Ashok Kumar for their patience and expertise in providing the means for this review. This article would not have been possible without their guidance and experience in muscle mechanics and biochemistry. Without a doubt, their words of inspiration and motivation were the very fuel behind this writing.

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Reviews in Undergraduate Research, Vol. 1, 21-28, 2002

Studies in Correlated Electron Systems

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In this survey of condensed matter physics, fundamental knowledge of the evolution and characteristic properties of the field will be discussed. Current trends in condensed matter physics will be identified along with their significance to the field. The reader will be exposed to research that is currently a work in progress in the sub-field of solid-state physics. The research concerns the system $Ce_{1-x}Y_xRhIn_5$ where heavy-fermion, antiferromagnetic behavior and superconductivity are being explored through measurements of electrical resistivity between temperatures of 1.8 K = T = 300 K. Future research and technological applications from the field of condensed matter physics are also identified and explored.

At the commencement of the 20th century, the foundations of knowledge of the macroscopic properties of matter were largely established. The fields of thermodynamics, elasticity, magnetism, and hydrodynamics collectively provided a thorough description of the "static and dynamic properties of gases, liquids, and solids at lengths long compared to molecular lengths" (Chaikin). In the 1920's, the discipline of "conventional solid state physics" emerged with the study of the quantum properties of solids (Chaiken). The areas of crystallography, elasticity, and magnetism were incorporated in the 1940's to define "solid state physics" in broader terms (Kohn). Accomplishments in the area of solid state physics have included the description of the quantum Hall effect, electronic band theory, and the detection of x-rays.

Two decades later, the 1960's, the science of condensed matter physics was introduced in an effort to study the physical properties of liquids, which resulted in an integration of the field of solid state physics (Kohn). Today, the discipline of condensed matter physics is defined as the

> …fundamental science of solids and liquids, states of matter in which the constituent atoms are sufficiently close together that each atom interacts simultaneously with many neighbors. It also deals with states intermediate between solid and liquid [e.g., liquid crystals, glasses, and gels], with dense gases and plasmas, and with special quantum states [superfluids] that exist only at low temperatures. All of these states constitute what are called the *condensed states* of matter (Panel on Condensed Matter Physics).

The evolution of this topic is important due to its radical advances and influences in "new experimental discoveries and techniques of measurement, control of the compositions and atomic configurations of materials, and new theoretical concepts and techniques" (Kohn). An example of a paradigm introduced by CMP is "the quantum-mechanical foundation of the classical sciences of mechanics, hydrodynamics, thermodynamics, electronics, optics, metallurgy, and solid-state chemistry" (Panel on Condensed Matter Physics). The technological innovations that are attributed to CMP include and are not limited to solid state lasers and lighting, superconducting magnets, and highly sensitive detectors of radiant energy (Panel on Condensed Matter Physics).

The discovery of superconductivity occurred in 1911 through the research of Kamerlingh-Onnes (Kresin, 4). Kamerlingh-Onnes discovered that the electrical resistance of mercury abruptly vanished at very low temperatures close to 4 K (Kresin, 4). Materials that exhibit superconductivity have a characteristic critical temperature T_C. Above this finite temperature, the material acts in its normal state and displays resistance, which is "the flow of electric current that accompanies the development of heat and the dissipation of energy" (Owens, 25). Below the T_C, the material enters a superconducting state where there is no electrical resistivity [material conducts electricity without

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losses] (Owens, 25). The characteristics of superconducting materials are not limited to the absence of electrical resistivity. They also include "anomalous magnetic, thermal, and other properties, so it is more precise to talk…(about superconductivity as) a peculiar state of matter observed at low temperatures" (Kresin, 5). In 1933, William Meissner showed that materials in their superconducting state displayed the property of perfect diamagnetism, the "complete expulsion of a weak magnetic field from the interior" (Kohn, S72). It was not until 1986 that high temperature (high T_C) superconductivity was discovered (Owens, 25). Today's studies have yielded high T_C's of up to 160 K, but the mechanisms behind this phenomenon remain to be a mystery- as does the "experimentalist's dream" of room temperature superconductors (Kohn, S73).

In 1964, the Japanese theoretical physicist Juan Kondo was successful in explaining the effect of a localized impurity spin on the scattering of conduction electrons at low temperatures (Kohn, S68). This phenomenon, now identified as the Kondo Effect, is briefly described in terms the Kondo temperature (T_K). At T_K, the conduction electrons in a metallic, non-magnetic material respond to the addition of a magnetic impurity atom by creating a magnetic shield that offsets the introduced magnetization. For $T \ll T_K$, the shield consists of a "singlet state with the conduction electrons" and causes a disappearance of the magnetic susceptibility (Kohn, S68).

The exploration of condensed matter energy states in the 1980's contributed to the interest in a new class of metals and systems called heavy-fermion systems. These materials are depicted as possessing electronic states that have "characteristic energy orders of magnitude smaller than [those] in ordinary metals " (Fisk/Ott). Numerous heavy-fermion systems are "intermetallic compounds in which one of the constituents is a rare-earth or actinide atom, with partially filled 4f- or 5f- electron shells" (Fisk/Ott). At high temperatures, heavy-fermion materials tend to behave as if the f-electrons are localized in their atomic states like conventional paramagnets in which the moments are random and tend to want to line up with the induced magnetic field (Fisk/Ott). At low temperatures, some of the f-electrons of the heavy-fermion system tend to become itinerant and order spontaneously with mostly antiferromagnetic- moments align by periodically alternating directions-, or less frequently ferromagnetic-moments align parallel-, properties (Fisk/Ott).

On an elementary level, the building blocks of condensed matter are formed through the logical combining of "electrons and nuclei to atoms and molecules" in large quantities ($\sim 10^{24}/cm^3$) (Panel on Condensed Matter Physics). The crystal lattices of these systems are considered to be either periodic or non-periodic. Periodic crystal lattices occur for simple systems of crystalline solids on an atomic scale (Kohn, S69). Non-periodic lattices are easily attainable in extensive systems where defects occur within in the materials due to fluctuations in temperature (Kohn, S68). Substitution alloys compose a class of relatively non-periodic systems and are denoted by the generic formula A_xB_{x-1} (Kohn, S69-70). The elements *A* and *B* are selected such that the elements possess identical valences [number of electrons occupying the outermost energy levels], matching crystal structures [in their pure form], and similar atomic radii (Kohn, S69-70). The Experimental section of this paper will utilize the substitution alloy relationship by discussing the elements Cerium and Yttrium in experimental context.

Recent Discoveries

Superconductivity of Buckyballs

Experimental research in the area of condensed matter physics during 1990 led to the discovery of buckyballs (Levi, 15). A buckyball is a spherical molecule composed of 60 carbon atoms that is easily manufactured in large quantities through simple techniques (Levi, 15). By 1991, "a crystal of C_{60} molecules had been found to superconduct when doped with alkali metals atoms, which cede electrons to the C_{60} lattice" (Levi, 15). Doping results in a material that either adds extra electrons to the material (which is then called N-type for the extra negative charge carriers) or creates "holes" in the material's crystal structure (which is then called P-type because it results in more positive charge carriers). In January of 2001, Jan Hendrik Schön, Christian Kloc, and Bertram Batlogg of Bell Labs, Lucent Technologies, discovered a new method of "injecting holes directly into the top layer of a C_{60} crystal without adding any ions to it" (Levi, 15). In addition, the experimenters explored the behavior of the buckyball crystal by varying a continuous doping level from positive to negative values (i.e. adding less holes, positive charges, to a material and increasing the number of electrons to yield a more negative charge).

The endeavors by the Bell Labs trio led to the discovery that the hole-doped (positive charged) C_{60} crystal had a maximum critical temperature of 52 K at a doping level between 3.0 and 3.5 holes per molecule (Levi, 15). In comparison to the negative charge injection near 3 electrons per molecule, the critical temperature (T_C) of the C_{60} material was only 11 K (Levi, 15). These results are important to the study of superconductivity in buckyballs for two main reasons: 1. the peaking of the T_C for negative charge and hole doping of three electrons per molecule or three holes per molecule emphasizes that superconductivity favors the

structure of 3 electrons per molecule, or A_3C_{60} where A is an alkali atom; 2. "hole doped C_{60} crystals turned out to be better superconductors than their electron-doped cousins" with much larger ranges of superconductivity (Levi, 15).

Continued research on the carbon-60 materials has led experimentalists to the realization that doping with larger atoms tends to expand the crystal lattice (Levi, 15). The resultant expansion thus causes broader spacing which "further reduces the overlap between the electron bands of adjacent molecules and narrows the bandwidths" (Levi, 15). The T_C affects the lattice through an inverse relationship with the bandwidth, the larger the doping atom, the higher the T_C (Levi, 15).

In January of 2001, the primary objective of the Bell Labs trio was to "incorporate interstitial ions that [would] expand a hole-doped C_{60} lattice" and hopefully raise the T_C well above 100 K (Levi, 15). In a time period of just nine months, the team was successful in accomplishing their goal as the T_C of the carbon-60 crystal was made to superconduct at a temperature of 117 K. The researchers were successful is raising the T_C as a result of a new approach, electronic hole-doping with "neutral molecules to [assist in] expand[ing] the crystal [lattice]" (Levi, 19). The neutral molecules, tribromomethane ($CHBr_3$), caused an expansion in the cubic lattice constant from 14.16 Å to 14.43 Å and a T_C of 117 K (Levi, 21). (A cubic lattice is generally described in terms of a unit cell with a cubic structure. The cubic structure includes three primary lattice vectors of equal magnitude (i.e. equal sides) and angles of 90° between each edge. The magnitude of the lattice vectors (i.e. length of each side) is referred to as the lattice constant.) Comparatively, when $CHCl_3$ was used as a neutral molecule, the T_C was only 80 K (Levi, 21). Experimentalists at Bell Labs are now searching for other neutral molecules that will expand the lattice constant in order to reach superconducting critical temperatures of 150 K (Levi, 21). If feasible, the future of thin-film electronics will benefit immensely (Levi, 21). Overall, the discovery of superconducting buckyballs is important, according to Robert Cava of Princeton University, because it means that thermodynamics is the only thing that has prevented physicists from unearthing more 100 K plus superconductors (Levi, 21).

Ferromagnetic Superconducting Material

A superconducting state is produced through lattice vibrations in low T_C materials or through magnetic fluctuations in high T_C materials that tend to push electrons together and expel any existing magnetic fields (Day, 16). Roughly one year ago, Gil Lonzarich's group at the Cambridge University and their collaborators at Grenoble's Atomic Energy Commissariat discovered that UGe_2, an alloy of uranium and germanium, "exhibited superconductivity and ferromagnetism simultaneously" (Day, 16). Now, another ferromagnetic superconductor, $ZrZn_2$, has been found to exist through the research of Christian Pfiederer of the University of Karlsruhe (Day, 16).

The compound $ZrZn_2$ is interesting because each component is paramagnetic, but together the system displays weak ferromagnetism with a Curie temperature, T_m, of 25 K (Day, 16). This point marks the "highest temperature at which magnetic order prevails" (Day, 16). When Pfiederer studied the samples of $ZrZn_2$ at varying pressures, he found that the compound had a superconducting transition at ambient pressure and at pressures lower than the critical pressure (Day, 17). The temperatures T_C and T_M were found to fall linearly in pressure from their ambient values until each vanished at the same critical pressure of 2.1 GPa (gigapascals) (Day, 17). The data presented above shows "strong evidence that the same electrons mediate both superconductivity and ferromagnetism. The high critical pressure indicates that superconductivity in $ZrZn_2$ occurs firmly in the ferromagnetic state, rather than close to the quantum critical point" (Day, 17). The study of ferromagnetic superconducting systems is hoped to lead to scientists to understand the phenomenon behind the high T_C superconductors (Day, 16). Also, the information gathered from the UGe_2 and $ZrZn_2$ systems strongly suggests that superconductivity is no longer an isolated phenomenon, but a generic effect caused by magnetic fluctuations (Day, 18).

Experimental

What and Why

Recently, it was discovered that heavy-fermion systems exist in Ce-based compounds with the structure $CeRhIn_5$ (Thompson). Specifically, the compound $CeRhIn_5$ is a heavy fermion system which displays antiferromagnetism but does not superconduct at ambient pressure. The Maple Lab at the University of California at SanDiego is currently exploring the compound $CeRhIn_5$. The nonmagnetic element Yttrium, Y, was added to the compound, $CeRhIn_5$ to yield the compound $Ce_{1-x}Y_xRhIn_5$, thus diluting the Ce magnetic moments. The element Yttrium was chosen because of its nonmagnetic properties and ability to be a substitution alloy in conjunction with the element Ce. Yttrium and Cerium are substitution alloys for three reasons: 1. each possesses three valence electrons (Cerium possesses 3 valence electrons at ambient room temperature and pressure. However, when subject to cooling or compression, the valency changes from about 3 to 4.); 2.

the atomic radii are similar with Ce=2.7Å and Y=2.27Å; 3. in their pure form both Ce and Y have a close packed hexagonal structure. The objective of studying the system $Ce_{1-x}Y_xRhIn_5$ with $0 \leq x \leq 1$ was to explore the magnetic phase diagram, and to search for possible non-Fermi liquid behavior at a quantum critical point where the Néel temperature, T_N, is suppressed to near 0 K or very low temperature ranges. The Néel temperature is the critical temperature of an antiferromagnetic material above which paramagnetism occurs.

Experimental Details

Single crystals of the intermetallic compound form $Ce_{1-x}Y_xRhIn_5$ were prepared and grown using a molten In flux technique in alumina crucibles, which were sealed under vacuum in quartz tubes. The molten Indium flux technique requires that the Indium surround the starting materials (with the higher melting temperatures on the bottom) inside the crucible. As the low-melting materials melt they flow over the higher-melting materials and incorporate them into the melt. The crucibles were heated inside a furnace to a temperature of ~1100°C for 24 hours and then cooled slowly (at ~5°C/hour) to 600°C. The samples were then removed from the furnace, immediately inverted, and centrifuged at room temperature, in an effort to remove the molten Indium.

In order to ensure the correct structure of each sample, powder diffraction x-ray data was taken. The peaks from the experimental data were then plotted and compared against those of the known theoretical $CeYRhIn_5$ and Germanium peaks. Once all of the major peaks from the sample were accounted for, it was concluded that the correct crystal lattice structure had formed. If significant peaks existed in the experimental data that were not categorized as concentrations of In, Ge, or $CeRhIn_5$, the sample was assumed to contain impurities. The lattice constants for each compound were determined by comparing the angles of the experimental peaks to the indices and angles of the simultaneous theoretical peaks. Then XLAT, a Least Squares (LSQ) program, used the resultant theoretical indices and experimental angles to calculate the precise refinement of lattice constants (*a, b, c)* in units of Angstroms. The lattice constants for the $Ce_{1-x}Y_xRhIn_5$ data were calculated for a tetragonal lattice system where $a = b \neq c$.

Resistivity was measured as a function of temperature in a ^{4}He cryostat. (Usually neutron diffraction measurements are necessary to determine the magnetic structure of the compound. Resistivity provides us with a hint, as we can postulate that there might be a Néel temperature if we see a kink.) The resistivity slices were prepared by attaching gold leads to the sample with silver epoxy. Temperatures in the range of 1.8K to 300 K were generated for each resistivity slice (preferably long, thin rectangular crystals of the sample) within the $Ce_{1-x}Y_xRhIn_5$ system being studied. Once the resistivity slices were placed into the ^{4}He cryostat, a magnetic field of 300 Oe was applied to overcome Indium's superconducting state and a maximum current flow of 10mA with a 16 Hz frequency was produced in order for the resistivity slice to reach low temperatures without interference.

Results

3.1 Crystal Formation

X-ray diffraction measurements reveal whether or not a specific compound of the type $Ce_{1-x}Y_xRhIn_5$, $0 = x = 1$, has formed the correct tetragonal crystal structure. The graphical representations of the x-ray diffraction measurements for $Ce_{.1}Y_{.9}RhIn_5$ are located in figure 1 and 2, with 2 representing a zoomed view. The plot consists of experimental $Ce_{.1}Y_{.9}RhIn_5$ and theoretical Ge, $CeRhIn_5$ data for intensity (counts) vs. angle (θ). The peaks in the plots result from variations in intensity where "for only certain values of θ will the reflections from all parallel planes add up in phase to give a strong reflected beam"- specular reflection (Kittel). A calibration standard, Germanium, was used to reveal phase shifts in the experimental peaks relative to $CeRhIn_5$ by mixing powdered Germanium with the powdered sample, x-raying the two substances, and comparing the known location of the theoretical Ge peaks with those from the experimental sample. Upon a close analysis of Figure 1 it is apparent that all of the major experimental peaks are accounted for by the theoretical data. Hence it can be easily determined that the sample compound $Ce_{.1}Y_{.9}RhIn_5$ formed the correct tetragonal crystal lattice structure. Similar processes and graphical

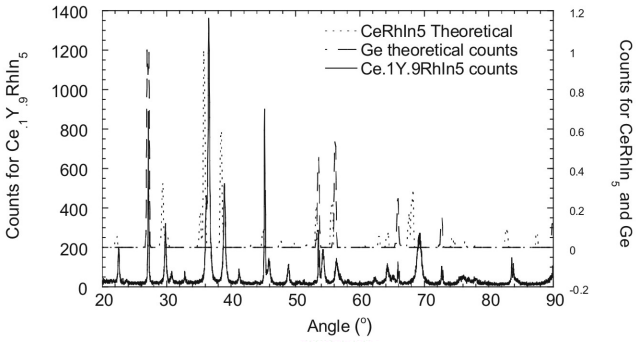

Figure 1. Graphical representation of the x-ray diffraction measurements for $Ce_{.1}Y_{.9}RhIn_5$ against $CeRhIn_5$ theoretical and Germanium theoretical. The graph is plotted as intensity vs. theta for $20° \leq \theta \leq 90°$.

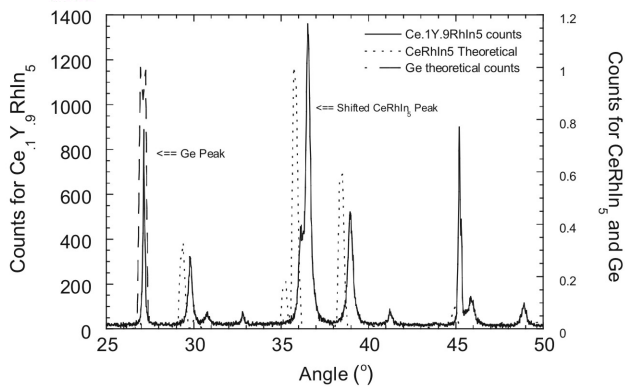

Figure 2. Magnified version of the graphical representation of the x-ray diffraction measurements for $Ce_{.1}Y_{.9}RhIn_5$ against $CeRhIn_5$ theoretical and Germanium theoretical. The graph is plotted as intensity vs. theta for $20° \leq \theta \leq 50°$.

representations were used to ensure that each compound of the $Ce_{1-x}Y_xRhIn_5$ system formed the tetragonal crystal structure.

3.2 Crystal Parameters

The x-ray diffraction measurements reveal that the crystals formed in the tetragonal $CeRhIn_5$ structure. The lattice constants for the $Ce_{1-x}Y_xRhIn_5$ system are *a* and *c*. These are plotted as a function of x, Yttrium concentration, in figures 3 and 4. A linear relationship should exist between the concentration of the substitute element and the size of the lattice parameters, which is called Vegard's Law. The direction of the linear relationship, increasing or decreasing, depends upon the system being analyzed. The graph for lattice constant *a* follows the rules set forth in Vegard's law. Figure 3 shows that as the concentration of Yttrium is increased, lattice constant *a* decreases, implying the cell is contracting along the *a* axis.

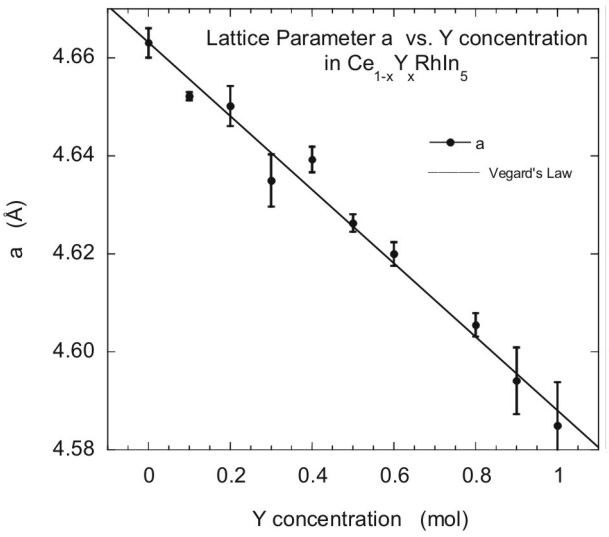

Figure 3. Tetragonal lattice constant a as a function of Y concentration x for the $Ce_{1-x}Y_xRhIn_5$ system. The dotted line is a fit to the data and the solid line represents the linear relationship of Vegard's law.

The graph for the *c* lattice parameter is shown in Figure 4. The relationship between the size of the *c* parameter and the concentration of Yttrium does not obey Vegard's law. Instead, the curve appears constant for $0 \leq x \leq 0.3$, and then a decreasing linear dependence occurs on x for $x \geq 0.3$. Overall, the results from Figures 3 and 4 provide evidence for a contracting cell structure along the *a, b, c* axes (where $b = a$) as the larger Yttrium ion is substituted on the Cerium site.

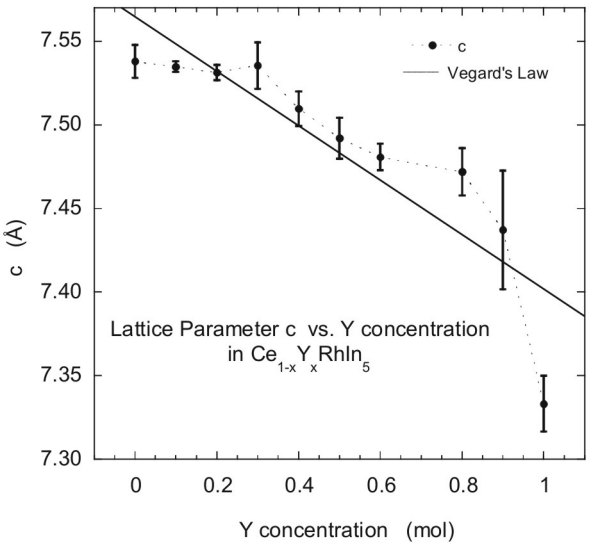

Figure 4. Tetragonal lattice constant c as a function of Y concentration x for the $Ce_{1-x}Y_xRhIn_5$ system. The dotted line is a fit to the data and the solid line represents the linear relationship of Vegard's law.

3.3 Electrical Resistivity

The normalized electrical "resistivity," $\rho/\rho(300K)$ vs. T of various $Ce_{1-x}Y_xRhIn_5$ compounds is displayed in Figures 5 and 6. Figure 5 compares the resistivity for concentrations of $.2 \leq x \leq .8$ while Figure 6 displays that of x=1 and x=. 9. The resistivity for $.2 \leq x \leq .8$ are weakly temperature dependent for temperatures above ~100 K. Below ~100 K a considerable decrease in slope tends to occur with a hump around ~50 K. For T<50 K there is a rapid decrease in $\rho(T)$ with decreasing T. This decline is caused by the onset of

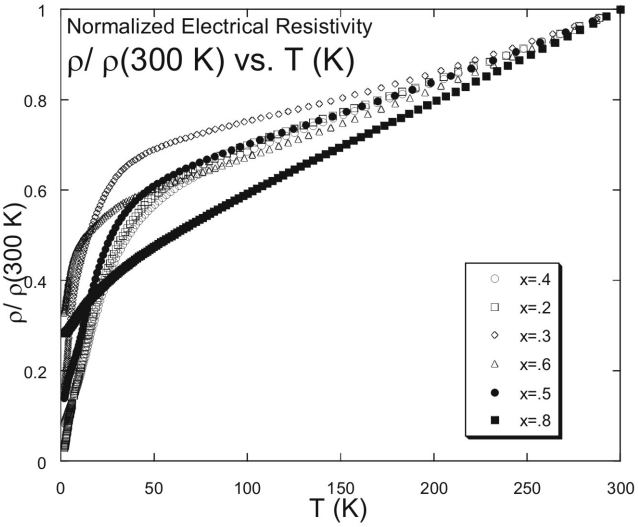

Figure 5. Electrical resistivity ρ normalized to its value at 300 K, ρ/ρ(300 K), as a function of temperature for $Ce_{.1}Y_{.9}RhIn_5$ with .2 ≤ x ≤.8 and x = .8.

coherence in the Cerium ion sublattice. The end member compound $CeRhIn_5$ exhibits similar behavior to the concentrations of .2 ≤ x ≤ .8. Upon close examination of the slopes in Figure 5, there is no consistency, as the slopes do not decrease nor increase with increases in the concentration of Yttrium.

The concentrations of x displayed in Figure 6 exhibit behavior that is different from those of the other x concentrations. The resistivity of the curves for x=0.9 and x=1 are weakly temperature dependent for temperatures above ~100 K. Below ~100 K a rapid decrease occurs in

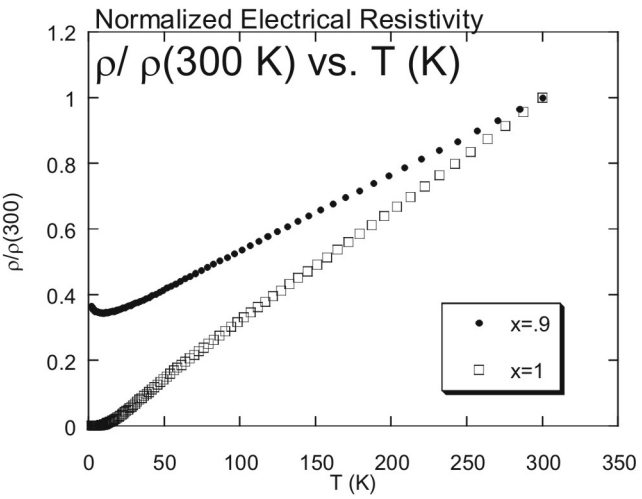

Figure 6. Electrical resistivity ρ normalized to its value at 300 K, ρ/ρ(300 K), as a function of temperature for $Ce_{.1}Y_{.9}RhIn_5$ with x = .9 and x = 1.0.

ρ(T) with decreasing T. A minimum occurs at ~20 K for x = 0.9 and then a slight increase in resistivity occurs for T< 25K. For x=1, $YrhIn_5$, ρ(T) decreases rapidly until ~50 K and then begins to level off slightly for T < 50 K. The graphs of resistivity for 0≤ x ≤ 1 do not display the Néel temperature where the onset of antiferromagnetism may occur as in some systems a kink is seen in the resistivity at the Néel temperature. It is suspected that antiferromagnetic ordering will occur for T< 1.8 K. The graphs do reveal the presence of the Kondo effect. Figure 5 displays an increase in resistivity, upturn in the curve, for T< 1.8 K while Figure 6 reveals a definite increase for x = 0. 9. There are no magnetic ions in YRhIn5, x = 1.0, which result in a lack of the Kondo effect. There is no presence or implication of superconductivity revealed in the resistivity data for 1.8 K ≤ T ≤ 300 K. The samples with x = 0.4, 0.5, 0.6 have linear ρ vs. T relationship, whereas Fermi-liquid theory predicts a T^2 temperature dependence of the resistivity. The possibility of non-Fermi liquid behavior in these samples needs to be explored further with specific heat and magnetization measurements.

Conclusion

The structure of the $Ce_{1-x}Y_xRhIn_5$ system was characterized by tetragonal powder diffraction x-ray measurements. The lattice parameters of this system were shown to decrease with increasing concentrations of Yttrium under ambient pressure and temperature. This provides evidence for a contracting cell structure. Measurements of electrical resistivity for 1.8 K = T = 50 K provide evidence of the Kondo effect. The experimental results offer no evidence of superconductivity.

Further investigation of the $Ce_{1-x}Y_xRhIn_5$ system should include tracking the suppression of the Néel temperature with increasing Yttrium by means of specific heat and neutron scattering measurements. Also, the physical properties of resistivity, specific heat, magnetic susceptibility, and nuclear magnetic resonance should be investigated at low temperatures in the vicinity of the quantum critical point, T_N, to determine if non-Fermi liquid behavior is observed. A detailed investigation of these systems should enhance our understanding of the interplay of magnetism, superconductivity, and non-Fermi Liquid behavior in correlated electron system.

Future Possibilities for Research and Application

Polymeric Carbon-60

Recently, a collection of experiments has revealed ferromagnetic behavior in polymerized C_{60} at temperatures near 500 K (Levi). Scientists are skeptical of this discovery because the "constituent molecules have no magnetic moments" (Levi, 18). Further investigation into this system is necessary in order to explain how and why a system composed solely of carbon can be magnetic if there are no unpaired electrons (Day, 18). Other research interests in carbon lie in the possible industrial aspects of the material. Companies are interested in finding a molecular-based material that can be cheaply manufactured and easily modulated (Levi, 18). However the samples and testing are costly (Levi, 18).

Solid State Lighting

A dilemma that is becoming more widespread every year is the challenge of generating electricity. The process is becoming more costly economically and environmentally as the years progress. The current state of lighting focuses mainly on incandescent and florescent sources (Bergh, 42). However, lighting applications that use Light Emitting Diodes (LEDs) and Organic Light Emitting Diodes (OLEDs), commonly referred to as Solid-State Lighting (SSL), are being developed. In SSL, a semiconductor converts electricity to light. The life of the device depends on packaging considerations, drive current, and the operating environment (increased temperatures produce lower light output). SSL offers economic and environmental savings over the current incandescent sources through long life, high efficiency, low voltage, and vibration and shock resistant. It is proposed that with SSL the US would be able to reduce lighting expenditures between 2000-2050 by $100 billion and spare 28 million metric tons of carbon emission into the atmosphere annually (Bergh, 42).

Other benefits of SSL include variety, aesthetics, and durability. The circuitry of SSL allows for control over the size, color, intensity, and design, thus resulting in light coloring similar to that of the sun and allowing physical features such as "flat packages of any shape that can be placed on floors, walls, ceilings, or even furniture" (Bergh, 42). Overall, SSL offers consumer friendly advantages that will revolutionize light sources over the next few decades.

About the Author

Kristen Rogers is a junior at Emory University where she is pursuing a bachelor of arts and science in both physics and mathematics. Upon graduation in May 2003, Kristen intends to pursue a PhD in one of the areas of experimental physics. Her final career goal is to develop national security technology for the United States Navy.

The research that Kristen conducted during the summer of 2001was completed through the REU program offered at the University of California at San Diego. There, she worked in the Maple Condensed Matter lab under the supervision of professor M. Brian Maple and graduate student Vivien Zapf.

Kristen concentrated her interests in low-temperature solid-state physics. The focus of her research was on the heavy fermion system $Ce_{1-x}Y_xRhIn_5$. This compound was of particular interest due to its display of antiferromagnetic behavior and lack of superconductivity at ambient pressure. Kristen participated firsthand in preparing the samples of the compound $Ce_{1-x}Y_xRhIn_5$ with varying concentrations of Ce and Y. She also acquired experience in measuring the magnetic properties of these compounds at temperatures as low as 1.8K and in magnetic fields of approximately 300 Oe. The ultimate goal in studying $Ce_{1-x}Y_xRhIn_5$ was to determine if the same antiferromagnetic, heavy fermion activity would be exhibited as that in the compound $CeRhIn_5$ when slowly adding Y and subtly eliminating Ce to the compound. The results, thus far, imply that antiferromagnetic behavior does exist and competition occurs at low temperatures between the Kondo effect and antiferromagnetism. This project is currently undergoing extensive research at UCSD. However, Kristen will no longer be an active participant due to academic obligations in Atlanta.

Acknowledgements

I would like to thank Professor M. Brian Maple, UCSD Physics Department, and the National Science Foundation 2001 Research Experience for Undergraduates program for enabling me the opportunity to partake in innovative, exciting physics research. I would also like to thank each member of the Maple lab for his/her kind support. In particular, I am especially grateful to Vivien Zapf for all the time and patience she devoted to my education, equipment know-how, and pestering questions.

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Reviews in Undergraduate Research, Vol. 1, 29-37, 2002

Genetic engineering of embryonic stem cells via site-directed DNA recombination

Anna Norman[1], Dr. Mark MacInnes[2]

This article reviews recent advances in genetic engineering of mammals utilizing DNA recombination techniques to produce targeted genome modifications. The general objective of these technologies is to discover novel gene functions via manipulation of gene expression, regulation, or encoded protein sequences. The advantage of gene site-directed DNA recombination is that the engineered variant remains in the normal context of its chromosomal locus. This feature is especially important for studying gene function in the context of its regulation in animal development. The target gene can be subject to both gain of function or loss of function mutations. In addition to precisely crafted modifications of single genes, alternatively, site-directed DNA recombination can also produce chromosomal changes including segment deletion, or inversion, or loss of heterozygosity between a homologous chromosome pair. Site-directed recombination is accomplished by certain mechanisms of DNA exchanges that were first discovered in bacteria and their viruses (bacteriophages). We will illustrate how these systems permit specific modification of the mammalian genome. Recombination enzymes of the integrase family such as the Cre protein (cyclization recombinase) have a well-characterized site-specific recombination mechanism. Cre recombinase catalyzes DNA strand exchanges in palindromic DNA target sequences called the locus of crossover (lox site). The biochemical flexibility of Cre interactions with lox sites permits a novel approach to mammalian gene targeting. For example, lox site orientation and-or change of sequences can modify the specificity of DNA exchange in fascinating ways.

This approach has been most successfully adapted for site-directed recombination in mouse embryonic stem cells (ES cells). For in vivo analyses, ES cells can be implanted into embryos to contribute in utero to the germline tissues. In progeny chimerical mice, the novel genetic trait may be transmitted via sperm or egg to offspring. ES cells also differentiate in vitro into numerous cell types allowing direct assessment of cell lineage phenotypes. Some of the differentiation properties of mouse ES cells in vitro have been confirmed in both human and primate ES cells. An understanding of ES cell genetic engineering and its potential applications is therefore of critical medical and ethical importance. Despite the successes of these approaches in murine ES cells, site-directed recombination technology has unresolved questions about its utility for human genetic and tissue therapy.

DNA Recombination in Embryonic Stem Cells

In the present review, we will focus on technologies for targeted DNA recombination in mouse embryonic stem cells (ES cells). These primordial stem cells are derived from pre-implantation embryos of the blastocyst stage (Evans and Kaufman, 1981; Martin, 1981). ES cells have the unique property of *pleuripotency*, that is the ability of the cells to differentiate *in vivo* into all subsequent tissues that arise from within the embryo (reviewed in Smith, 2001). Upon injection of ES cells into host blastocyst stage embryos, the ES cells contribute *in utero* to embryonic development of all of the somatic and germinal tissue lineages. A novel line of mice is established when a novel genetic trait is transmitted in the germline of the founding progeny animals . To date, there is a literature of *several thousand* genetically novel lines of mice created from ES cells.

ES cells also exhibit remarkable proficiency to differentiate *in vitro*, providing researchers with many derivative cell types for direct genotype to phenotype

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analyses (O'Shea, 1999). Other mammalian cell systems that are permissive for targeted DNA recombination include chicken DT40 cells (Dhar et al., 2001; Winding and Berchtold, 2001) and Chinese Hamster ovary (CHO) cells (Thompson and Schild, 2001). Recently, some of the characteristics of pleuripotency found mouse ES cells *in vitro* have been replicated with both human and monkey ES cell lines (Cibelli et al., 2002; Reubinoff et al., 2001; Reubinoff et al., 2000). These findings highlight the crucial medical relevance and ethical implications of ES cell genetic research regarding the genetic manipulation of human embryonic stem cell lines.

Complementary Strategies for Targeted DNA Recombination in ES Cells

Altering target genes within ES cells employs two complementary strategies: homologous recombination and integrase mechanisms. Homologous recombination (HR) entails double reciprocal exchanges between DNA molecules dependent largely on overall sequence identity. HR has long been used to introduce exogenous DNA sequence into a similar target sequence within mouse ES cells (Thomas and Cappechi, 1986). In ES cells, HR coupled with appropriate selection using antibiotics for the desired recombinant cells provides an efficient method to produce gene-targeted mutations. Selected cells (clones) derived from single progenitor cells must be screened for HR by molecular methods (primarily polymerase chain reaction - PCR) to identify those with the desired genetic modification. The mechanism of HR within mammals has a complex biochemistry (Thompson and Schild, 2001). In contrast, prokaryotic DNA recombinases provide a complementary and less complex avenue towards site-directed genetic recombination. These mechanisms require much less sequence identity than HR, decreasing the amount of foreign DNA that is incorporated. In addition, while the best characterized DNA recombinase enzymes are of bacterial and yeast origin, they efficiently catalyze recombination in mammalian cells.

The detailed biochemical understanding of prokaryotic DNA recombinases and their substrate target sequences has allowed researchers to efficiently create specific cell type, or developmental control of the timing of genetic alterations in mice. This feat is accomplished by placing Cre recombinase under the control of a cell-type-specific gene promoter with the desired pattern of expression (reviewed in Sauer, 1998). Another advantage of site-directed DNA recombination is that DNA exchange is targeted by certain short oligonucleotide DNA sequences (e.g. *lox* sites) pre-positioned via HR in the mouse chromosome. In the specific case of DNA insertion, recombination sites in the plasmid vector identical to those in the genome provide the substrate for plasmid to chromosome recombination-mediated insertion of sequence. Variations in *lox site* targeting allow repeated exchanges of genetic material at the same mammalian genome site, or alternatively, into separate sites in the same genome.

A caveat to this enthusiastic introduction to site directed DNA recombination is the recent discovery of Cre recombinase-mediated genotoxicity in the form of chromosome aberrations (Loonstra et al., 2001; Thyagarajan et al., 2000). Characterization of this untoward consequence of recombinase activity has led to successful efforts to minimize its confounding effects on site-directed recombination studies in cells, *in vitro* and in tissues, *in vivo*. We will briefly survey potential applications of these technologies for human gene therapy in the context of therapeutic cell type rep*lac*ement derived from human ES cells. In a related topic not covered here, others have recently reviewed advances in molecular methods of assembling gene targeting vectors by HR in *E. Coli* bacterial artificial chromosome vectors (BACs) (Copeland et al., 2001).

The CRE/*lox* Site Recombination Mechanism

The integrase family of DNA recombinases shares the biochemical feature of bimolecular reaction kinetics whereby the enzyme recognizes a specific DNA recombination sequence. Members of this family include Cre recombinase from bacteriophage P1, bacteriophage lambda integrase, the yeast Flp recombinase, and the bacterial XerCD recombinases. These enzymes accomplish DNA strand exchange in a two step process between DNA substrates. One pair of strands is exchanged to form a recombination junction intermediate that does not move, while the second pair of strands is then exchanged during resolution of the junction. Van Duyne (2001) reviewed the structural biology of these recombinases with emphasis on the crystal structures of Cre with its DNA substrate (van Duyne, 2001). Cre recombinase and to a limited extent Flp recombinase (Seibler et al., 1998) have been used for enzyme mediated site-directed DNA recombination. We will describe in some detail how Cre recombinase interaction with its substrate DNA can be altered in ways that produce diverse genetic outcomes in mammals.

The Cre protein (38 kDa) is encoded by the *E. Coli* phage P1. P1 is maintained inside *E. Coli* cells as a single copy, circular DNA plasmid molecule. The role of Cre protein is to exchange and separate copies of P1 that arise after its replication in order to allow partitioning of the two P1 molecules at each cell division (Hoess and Abremski, 1984; Sternberg and Hamilton, 1981). The target site of Cre is the *loxP* sequence of 34 base-pairs (bp), containing two 13 bp inverted repeats flanking an 8 bp core sequence (Figure 1). Two Cre molecules bind to each *loxP* site, one on each half of the palindrome (van Duyne, 2001). Cre molecules bound to DNA then form a tetrameric complex bringing two *lox* sites into proximity. The Cre-mediated strand cleavage and exchange between *lox* sites occurs following the first bases and before the last base of the 8 bp cores. (The reader is referred to van Duyne (2001) and references therein for beautiful crystal structure representations of this complex). The DNA strand asymmetry of the 8 bp core also confers directionality on the *loxP* site (Hoess and Abremski, 1984). *loxP* orientation determines the type of recombination that will occur between *loxP* sites. Cre recombinase catalyzes both inter-molecular DNA exchanges and intra-molecular excision or inversion (Figure 2). If two *loxP* sites in the same molecule are co-aligned, Cre recombination will preferentially produce excision and circularization of the DNA between the sites (Figure 2A) (Baubonis and Sauer, 1993; Sauer and Henderson, 1989). Cre also catalyzes the reverse reaction, the integration of DNA into a single *loxP* (Figure 2A). However, integration is quite inefficient since the inserted DNA is immediately flanked by duplicated *loxP* sites, which permit re-excision (Araki et al., 1997). When two *loxP* sites are inverted in orientation intra-molecular recombination will produce an orientation switch of the insert (an inversion) with a 50:50 probability (Figure 2B) (Hoess et al, 1986, Feng et al, 1999).

The Cre/*lox* system as outlined above can be used to introduce certain kinds of gene mutations as well as chromosomal inversions, truncations, or deletions (Zheng et al., 2000; Feng et al, 1999). Further, Cre-induced mitotic

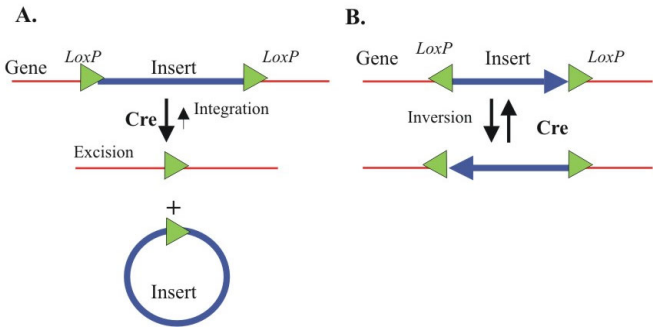

Figure 2. Cre mediated *loxP* recombination reactions at single *loxP* sites

(A) Homologous *loxP* sites flanking an insert recombine circularize the insert. B) Recombination between inverted *loxP* sites leads to a 50:50 probability of segment inversion.

chromosome recombination between single *loxP* sites on each member of a homologue pair has also been used to create genetic mosaics in mouse ES cells (Liu et al., 2002). This experiment simulates chromosome loss of heterozygosity (LOH) that is seen in many types of tumors. Cre-induced mitotic recombination in a tissue lineage *in vivo* would permit studies of LOH effects on development or in adult mouse tissues.

The recombination properties of Cre at a single *loxP* site select against the insertion of precise DNA segments into the target chromosome. Upon targeted integration of DNA, the *loxP* site is duplicated, leading to the highly favored intra-molecular excision (Fig. 2A). When an integration event does occur, not only is the DNA of interest integrated into the genome, DNA from the targeting plasmid vector is integrated as well. Alternative strategies have been devised using the Cre/*lox* system in order to create higher frequency and stability of insertion events, *in vitro*, and ultimately to eliminate plasmid DNA introduction into the genome.

Left hand palindrome *Right hand palindrome*

8 bp core

loxP ATAACTTCGTATA GCATACAT TATACGAAGTTAT

Figure 1. DNA sequence of wild type *loxP* site

The 13 bp inverted repeats (palindromes) flank an 8 bp asymmetric core sequence where the recombination exchange takes p*l*ace. One Cre recombinase molecule binds to each palindrome sequence (not shown). Strand cleavage positions are after the first, and before the last base of the 8-bp core.

CRE Recombination with Mutant *lox* Sites: Strategies for DNA to Chromosome Insertion

Albert et al. (1995) found that mutations in *loxP* permit integration of DNA at a plant target site in the plant genome while avoiding its immediate re-excision. This strategy was also successful for integrating foreign gene DNA into a mouse chromosome (Araki et al., 1997). A single mutant *lox* site in which nucleotides were altered in the right hand palindrome was pre-positioned by HR in the chromosome target (Figure 3). In the targeting vector, a distinct *lox* mutation was incorporated into the left hand palindromic element. The two mutant *lox* sites were in co-alignment and still enabled Cre to catalyze inter-molecular recombination in ES cells. However, the integration resulted in the creation of two *de novo lox* sites, one containing both left-end and right-end mutations, while normal *lox* P was generated at the other site. Cre poorly recognizes the LE+RE *lox* site, which inhibited re-excision between it and the *loxP*. This approach facilitated efficient insertion of a targeting cassette.

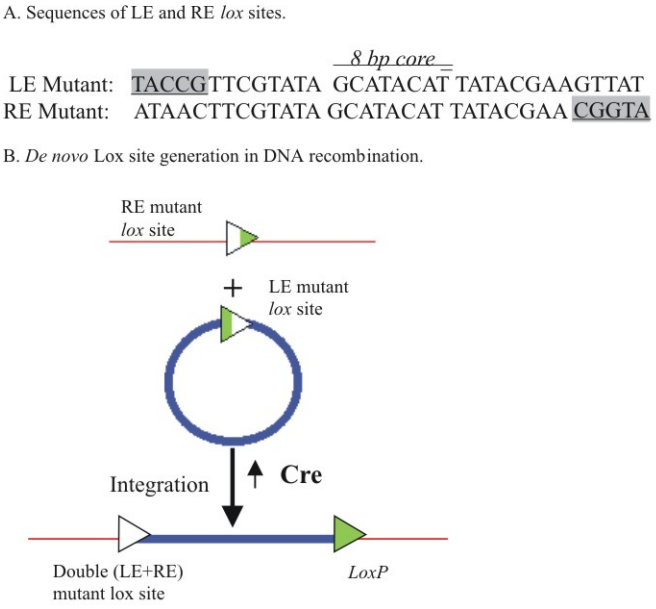

Figure 3. The LE/RE mutant *lox* site strategy for segment integration

Cre-mediated recombination between the mutant right end (RE) and left end (LE) *lox* sites produces a trapped product double mutant (LE+RE) *lox* site and a WT *loxP* site that are less susceptible to intra-molecular excision.

The idea that different *lox* sites may not recombine efficiently but that identical *lox* site recombination remains proficient led to an in-depth study of these interactions. Lee and Saito (1998) identified many mutant *lox* sites that recombine efficiently with an identical partner complex but not with *loxP* (Figure 4A). For example, *lox 2272* and *lox 2372* sequences contain two nucleotide changes in the core 8-bp sequence (Lee and Saito, 1998). The *lox* FAS site occurs naturally in *Saccharomyces cerevisiae* (Sauer, 1996). *Lox FAS* has a completely different consensus core sequence from *loxP* while remaining an efficient substrate for Cre. This fact illustrates the plasticity of *lox* sites. The *lox 511* site contains a single nucleotide mutation in the core sequence (Hoess et al., 1986). The recombination efficiency between homologous and heterologous pairs of *lox* sites has been studied in *E. Coli* (Siegel et al., 2001) (Table 1). Their results show that these heterologous pairs of *lox* sites undergo recombination at a much lower frequency than homologous pairs.

Siegel et al. (2001) used a green fluorescent protein (GFP) gene flanked by heterologous *lox* sites in the *lac* Z reporter gene (responsible for production of β-galactosidase) of a plasmid DNA. Before recombination, the bacterial colonies expressed GFP and emitted green fluorescence. Correct recombination events resulted in excision of the GFP gene and permitted the *lac* Z gene to be translated, producing a loss of GFP fluorescence and concomitant expression of functional *lac* Z gene. Such *E. Coli* recombinant colonies were non-fluorescent and blue dye colored on X-gal medium. The assay quantified the accumulated recombination events over many generations of colony growth (~18h) and therefore it was very sensitive to low levels of correct recombination. The assay also distinguished aborted or aberrant recombination repair products from true GFP excision via absence of correct *lac*Z gene activation. These findings indicated that recombination between homologous pairs of *lox* sites (whether similar or dissimilar to *loxP*) can occur efficiently *in vivo* while recombination between heterologous pairs occurs much less efficiently. Lee and Saito (1998) also noted the occurrence in some combinations of arrested intermediate recombination structures in their *in vitro* plasmid assay system. In these situations, recombination proceeded to exchange one DNA strand but, due to the heterozygosity of the *lox* sites, the intermediate wasn't able to resolve into the final recombination product. The potential persistence of arrested intermediates between heterologous *lox* sites may have implications for the use of the Cre/*lox* system in mammals (see below).

Given the low level of Cre-mediated recombination between several heterologous pairs of *lox* sites, new gene targeting techniques were developed that exploit this selectivity. The DNA to be inserted into the genome is constructed so that

Table 1. Recombination frequencies in *E. coli* among three mutant lox sites and loxP. Reprinted with permission of the authors. Note, Siegel et al (2001) reported recombination results for a sequence originally thought to be *lox2272*. However, upon inspection the published sequence it was not *lox2272* but rather *lox2372* (from Lee and Saito, 1998). (A. Bradbury, personal communication)

	WT	2272	FAS	511
WT	99.6			
2272	0.5	99.7		
FAS	0.2	1.7	99.4	
511	10.3	1.6	0.0	99.8

it is flanked by heterologous *lox* sites. The genomic target contains the matching *lox* sites by HR pre-placement. In the presence of Cre, during a double-reciprocal recombination event, there occurs a 50:50 probability of replacement of the *lox*-flanked chromosomal DNA by the targeting allele (Figure 4B). This exchange is referred to as recombinase-mediated cassette exchange, or RMCE. The RMCE system permits efficient insertion of *lox*-flanked DNA into the mammalian genome (Feng et al., 1999; Kolb, 2001; Trinh and Morrison, 2000). It is used to swap wild-type functional gene segments with knockout or otherwise mutated gene segments without incorporation of extraneous DNA.

Kolb used HR and site-specific RMCE to successfully insert a reporter gene into the mouse β-casein locus (Kolb, 2001). Kolb created a targeting construct consisting of *lox* 2272 and *loxP* sites flanking a selection marker that was integrated into the ES cells genome via HR. Recombination with a targeting construct containing a luciferase reporter gene flanked by *lox*2272 and *loxP* sites resulted in the efficient switching of the *lox*-flanked cassettes (Figure 4B). Typically, the HR step involves using selection markers such as geneticin (G418) or hygromycin resistance for positive selection placement of the *lox*-flanked gene segment. In RMCE, the selection marker is removed to avoid dysregulation of the modified allele. The loss of the selection marker by site directed recombination is tested by replica plating of cell clones in the appropriate selection medium.

CRE Recombinase Expression: Regulation in Mammalian Development

The Cre/*lox* system for genetic recombination also permits *lox*-flanked target gene alteration via stage- or tissue-specific control dependent upon the regulation of Cre gene expression *in vivo*. The majority of mammalian genes are thought to have developmentally regulated expression or they may express only in specific tissues. Because *lox* sites are quite short, their presence in the genome does not generally impair expression of their 'host' gene (Silver and Livingston, 2001; Trinh and Morrison, 2000). Often,

A. WT *loxP* and Mutant *lox* site sequences:

	8 bp core
loxP	ATAACTTCGTATA GCATACAT TATACGAAGTTAT
lox FAS	ATAACTTCGTATA TACC TTTC TATACGAAGTTAT
lox 2272	ATAACTTCGTATA GGATAC TT TATACGAAGTTAT
lox 2372	ATAACTTCGTATA GGATACC T TATACGAAGTTAT
lox 511	ATAACTTCGTATA GTATACAT TATACGAAGTTAT

B. Insertion of *floxed* sequences via double reciprocal recombination.

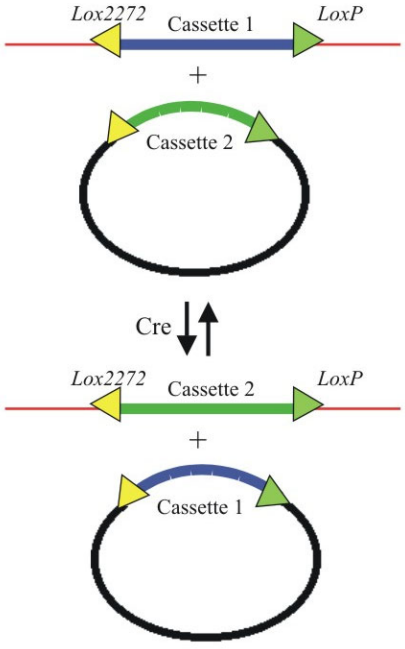

Figure 4. Recombinase Mediated Cassette Exchange (RMCE)

A) Sequence differences in mutant *lox* sites. B) Heterologous *lox* sites (in this example *loxP* and *lox* 2272) sites flanking an insert can be used to swap pre-placed genome cassette (Cassette 1) for a targeting insert (Cassette 2) that is flanked by the same *lox* sites. Recombination events that result in successful cassette exchange are assessed by molecular analysis or by antibiotic growth selection.

expression of a mutation in the whole animal rather than at a specific time or tissue location would prove lethal, thereby preventing the study of phenotypes of the gene (Schipani, et al., 2001). The targeted gene is flanked by *loxP* sites and then integrated into the genome via HR. The altered ES cells are then developed into mice with the *lox*-flanked gene intact. The Cre gene is engineered to express under the control of a cell-type-specific promoter, whichever suits the purpose of the study (Metzger and Chambon, 2001; Metzger and Feil, 1999; Schipani et al., 2001). A line of Cre tissue-specific expression transgenic mice is created separately and evaluated for cell-type specific Cre-expression. When the two transgenic mouse lines are mated, the progeny of doubly transgenic genotype will enable activation of Cre expression in the appropriate cell type or time of development. The mutation (usually an excision recombination) arises by Cre expression in most of the affected cells. Typically at low levels of Cre expression, some cells are mutated while others are not, creating genetic mosaicism in the tissue. Mosaicism may be useful for phenotype interpretation by providing modified and unmodified cells side by side.

In addition, it is important to assess whether Cre may cause untoward effects such as cell death arising from Cre expression in the transgenic parental and non-targeted littermate mice. Many transgenic mouse lines thought to have tissue-specific Cre-transgene expression appear normal outwardly and by histology (Lewandoski, 2001; Nagy and Mar, 2001). We shall see next why careful examination is warranted of tissue specific Cre-transgene expression.

CRE Recombinase Genotoxicity

While site-directed recombination is a useful tool for genetic manipulation, Cre recombinase is also inherently toxic to many mammalian cells lines. This toxicity is the result of the recombinase activity of Cre (Loonstra et al., 2001; Silver and Livingston, 2001). These researchers have reported total cessation of cell replication, cell death, and an abundance of chromosomal aberrations and aneuploidy following *high level* Cre recombinase expression. These events could be the result of illegitimate DNA recombination or strand breaks induced in the mammalian genome. Another observation consistent with this notion is that cells cultured in the presence of high levels of Cre showed an increase in the number of cells in the G2/M phase of the cell cycle (the period just before mitosis begins and mitosis itself). This result indicates that the DNA damage is severe enough to trigger marked G2/M cell cycle checkpoint arrest (for a RUR review of cell cycle check points, see the article by Renthal in this issue).

A corroborating discovery was that of the existence of pseudo-*lox* sites in the mammalian genome (Thyagarajan et al., 2000). Illegitimate mammalian genomic *lox* sites elicited Cre-mediated recombination. Indeed, Cre also induces recombination at secondary recombination sites that occur naturally in *E. Coli* and in yeast (called *lox*B sites) (Sauer, 1996; Sternberg and Hamilton, 1981). As Cre catalyzes apparent interaction between pseudo-*lox* sites in mammalian cells, these events could therefore result in deletions or other chromosome alterations. Consequently, Cre induced breaks at endogenous chromosomal sites may possibly complicate the interpretation of Cre/*lox* experiments.

The studies that elucidated this problem also offered possible technical solutions. Loonstra et al. used a hormone-regulated Cre gene that was expressed at negligible levels without induction (Loonstra et al., 2001). When cells were subjected to supra-basal but not saturating levels of the hormone, Cre expression was elevated sufficiently to catalyze excision of a *lox*-flanked reporter gene without inhibiting cell growth and without production of visible chromosome aberrations. In their work cited in Loonstra et al (2001) a moderate level of the hormone elicited complete excision of another *lox* flanked genomic target without apparent genotoxicity. Others have employed variations on 'hit and run' strategies utilizing a negative feedback loop to circumvent overt Cre genotoxicity (Pfeifer et al., 2001; Silver and Livingston, 2001). Cre expression vectors were engineered to produce low levels of Cre coupled with a genetic negative feedback loop to limit the amount of Cre in the cells. Silver and Livingston used a retroviral vector containing a Cre expression gene with a single *lox* 511 site in its LTR. This retroviral vector was engineered so that following its reverse transcription and genome integration, the Cre-expression vector contained duplicated LTRs with co-aligned *lox* 511 sites. When Cre was expressed at a level high enough to cause recombination between the *lox* 511 sites, the entire Cre gene was auto-excised removing further synthesis of Cre after a few cell generations. This strategy resulted in cells capable of targeted excision of *lox*-flanked sequences in an unlinked target gene. As Cre expression was limited, there were no observed genotoxic effects.

It is also likely that the amount of Cre expressed in ES cells in culture can be controlled simply during the gene transfer process. Linear DNA introduced into ES cells by the technique of electroporation is efficiently integrated

into the genome, either randomly or by HR, in ES cells. In contrast, circular plasmid DNA has approximately 8 fold lower probability of chromosome integration (Taniguchi et al., 1998). This difference can be exploited to control magnitude and duration of Cre-recombinase expression simply via the transient presence of Cre expression plasmid. In addition, a fluorescence reporter plasmid called 'Cre-Stoplight' has been developed recently to bioassay Cre recombinase activity in live cells by epifluorescence microscopy or flow cytometry (Yang and Hughes, 2001). The plasmid incorporates dual reporter gene cassettes containing a *lox*-flanked Discosoma coral fluorescent protein, DsRed, and a transcriptionally inactive green fluorescent protein (EGFP). When sufficient Cre is expressed in cells containing Cre-Stoplight the DsRed gene is excised and rendered inactive by virtue of its *flanking lox* sites. Then the upstream promoter is brought into apposition to the EGFP gene. Therefore, mouse ES cells taking up DNA after 72 hours show considerable fractions of cells (> 20 %) with both red and green epifluorescence caused by the switch of DsRed to GFP production (K. Nowak and M. MacInnes, unpublished observations). We are now investigating the utility of monitoring levels of Cre recombinase by transient expression of Cre Stoplight to obtain efficient GFP activation, and presumably recombination at specific genomic target sites. As indicated above, for *in vivo* experiments similar engineering of tissue-specific autoexcision, or autoregulation, of Cre transgene may help avoid the possibility of confounding non-specific genotoxicity in the developmental stage or tissue of interest.

Site-directed Recombination in Mammalian Functional Genomics and Human Gene Therapy

The Cre/lox recombination system and HR have given researchers powerful tools for investigating novel mammalian genes. Conversely, they can also be used to create controlled gross deletions, inversions, and chromosome mitotic recombination in order to characterize certain genetic disease processes. The rich applications of Cre/lox hold promise for elucidating thousands of novel gene functions, an essential integrative genetics component of the functional genomics / systems biology era. The recent completion of the draft sequences of both mouse and human genomes will greatly facilitate building HR and Cre/lox recombination vectors in both mouse and human cells. The production of gene targeting vectors for HR and Cre/lox strategies must be automated into a high-throughput enterprise in order to realize the full potential of these approaches (Copeland et al., 2001). Characterization of gene function in ES cells and their derivatives in vitro would facilitate preliminary genetic analyses without necessitating very costly and ethically questionable production of tens of thousands of new mutant mouse lines.

It is of great interest whether HR gene targeting is possible in human ES cells given that few or no diploid human cell lines have yet proved useful for HR and site-directed recombination. Recent provocative research produced successful isolation of stem-like cells from human and monkey embryos (Reubinoff et al., 2001; Reubinoff et al., 2000; Thomson and Marshall, 1998) and from parthenogenetically activated Macac eggs (Cibelli et al., 2002). Similarly, adult stem cells have been isolated from mammalian bone marrow, liver, pancreas and brain (for a review see Clarke and Frisen, 2001). These milestones raise controversial ethical possibilities that human cell therapy (and huES cell genetic engineering) may become a reality for numerous diseases with a genetic component. In theory, human stem cells could be 'corrected' through retroviral vector incorporation or via HR, and this approach complemented by Cre/lox genetics. Two major technical concerns in cell replacement therapy are, first, the possibility of implanted cell/tissue rejection. Ideally, this difficulty is circumvented by use of the patient's own (autologous) stem cells. The second technical problem concerns a significant possibility of a carcinoma arising from implanted stem-like cells. As illustrated in this brief review, we have shown how activation or reversal of targeted genetic modifications can be engineered using Cre/lox. This approach may offer opportunities to provide additional safeguards against neoplasia in therapeutic strategies involving cell replacement with human stem cells.

About the Author

Anna Norman is currently a junior at the College of St. Benedict in St. Joseph, Minnesota where she is majoring in Biology. After graduation, she hopes to attend either medical school or to enroll in a joint MD / PhD program. During the summer of 2001, she was accepted to a National Science Foundation sponsored Research Experience for Undergraduate students (REU) Program hosted by the Los Alamos National Laboratory. Under the mentorship of Dr. Mark A. MacInnes, a geneticist in Biosciences Division, she tested recombination efficiency of a homologous and heterologous pair of *lox* sites flanking

a marker gene introduced into mouse ES cells. Using recombinant DNA techniques, Ms. Norman assembled the plasmid vectors containing pairs of *lox* sites flanking a drug resistance marker gene. These vectors were then linearized and introduced into mouse ES via electroporation. Recombination efficiency was analyzed based on the number of colonies that grew, and cell growth rate, in culture medium containing antibiotics. Using this method she found resistance to intra-molecular recombination between *lox* 2372 and *lox* FAS but proficient as recombination as expected between *loxP* sites. During the experiment, quantifying colony yield and cell regrowth confirmed that certain toxic effects of Cre recombinase occurred in mouse ES cells leading in part to the discussion of Cre genotoxicity in this review. Improvement of methods outlined in the review is a major emphasis of the MacInnes laboratory at this time.

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Reviews in Undergraduate Research, Vol. 1, 38-43 , 2002

CP Violation and the Dominance of Matter

James Morris[1], communicated by Dr. Elliott Cheu[1]

The observable Universe reveals a clear dominance of matter over antimatter. If this asymmetry is consistent with the Universe as a whole, then a contradiction arises between observation and any naïve formulation of Big Bang theory. CP violation, violation of the combined C (charge conjugation) and P (parity) operators, is one of three necessary conditions of matter dominance theorized by Sakharov in 1967. Therefore, further study of CP violation is warranted to determine its role in the evolution of a matter-dominated Universe.

There are two ways by which violation might occur: direct and indirect. The first possibility is that physical eigenstates may be written as a linear combination of CP eigenstates – a small amount of the opposite CP eigenstate might cause CP violation. This violation from mixing of CP eigenstates is also called indirect CP violation. Independent of indirect violation, CP violation might also occur in the decay or direct CP violation. A famous experiment, which investigated the possibility of indirect CP violation, was conducted in 1964. Since then several experiments have been conducted to determine if CP violation also exists in the decay. This review summarizes the methodology of two such experiments.

Despite the progress made in understanding the nature of CP violation, the future of research of CP violation remains promising. Several proposed experiments involve determining the degree of CP violation in much more rare decays. We summarize one such future experiment of analyzing CP violation in these decays.

Finally, due to the rare occurrence of CP violation, future investigations will require high rate experiments with high efficiency detectors. To this end we test the efficiency of one such detector which may be used in such experiments. A high rate environment similar to that expected in proposed CP violating experiments was used to examine the detector's behavior. In particular, this detector was tested using electrons from K_L decays ($K_L \rightarrow \pi^+ e^- \nu_e$). The high efficiency of this detector in this high rate environment is a testament to the future viability of this and similar devices in the investigation of CP violation.

Matter Dominance

The Naïve Big Bang

Big Bang Theory is widely accepted among the scientific community due to its empirical merits. For this reason, scientists have begun to establish particulars and study predictions of the theory. One prediction is that the early Universe was devoid of matter, containing only energy in the form of photons. As the Universe expanded, matter was able to form through processes such as $\gamma + \gamma \rightarrow p + \overline{p}$ (where γ denotes a photon, p a particle, and $\overline{p}$ an anti-particle). As can be seen, these processes produce equal numbers of particles and anti-particles. Naïve formulations of the Big Bang, which consider these processes alone, would result in a Universe that is matter/antimatter symmetric. However, we observe matter, not antimatter, out to 10Mpc (Dolgov, 1997), and strong arguments can be made in extending this observation to the Universe as a whole. Therefore, a contradiction arises between the small ratio of antimatter to matter, which current estimates place at 10^{-4}, and a naïve formulation of the Big Bang (Steigman, 1976).

Baryogenesis

The vast majority of matter is comprised of particles called *baryons*. Baryons, such as protons and neutrons, are particles with three quarks. There are two ways by which the baryon (or matter) asymmetry in our universe can be explained: either the abundance of baryons is the result of some set of initial conditions or the abundance came about by some physical mechanism. In 1967 Sakharov showed that at least three conditions are required for such a mechanism to exist (Sakharov, 1967). The conditions are

1. Baryon number violation
2. Thermodynamic Nonequilibrium
3. C and CP violation.

If some mechanism satisfies these three conditions, the baryon dominance we see today may be created from an

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early Universe which consisted mostly of energy. This is commonly referred to as *baryogenesis*. This review will concentrate on the CP violating condition of baryogenesis.

CP violation and Baryogenesis

Quantum mechanically, CP violation implies that the system is not symmetric under combined operations of *charge conjugation* (C) and *parity inversion* (P). The operation of charge conjugation exchanges particles with anti-particles, and the operation of parity inversion changes the signs of the coordinates. The combined operation of C and P relates the rates of particle transitions to anti-particle transitions. If CP were conserved, then the rate for a particular particle decay would be the same as that for the related anti-particle decay. If CP were violated, these transition rates would differ. For example, if the transition rate from anti-particle to particle were greater than the transition rate from particle to anti-particle, then matter creation would be preferred over antimatter creation.

Neutral Kaon and CP conservation

CP violation was first discovered in decays of the K meson (a meson is a particle comprised of quark anti-quark pairs), also known as the kaon. Kaons have the unique propriety of being produced by the strong force and decaying via the weak force. This occurs because the kaon is the lightest particle with a strange quark. Strong decays preserve quark type, and all decays produce less massive particles. However, because the kaon itself is the lightest particle with a strange quark these less massive particles cannot contain strange quarks. Thus, the kaon cannot decay via the strong force but instead decays via the weak force, which allows quark "flavor" to change. Due to this phenomenon, one can write the weak eigenstates as superpositions of the strong eigenstates as

$$\left|K_1^o\right\rangle = \frac{1}{\sqrt{2}}\left(\left|K^o\right\rangle + \left|\overline{K}^o\right\rangle\right)$$
$$\left|K_2^o\right\rangle = \frac{1}{\sqrt{2}}\left(\left|K^o\right\rangle - \left|\overline{K}^o\right\rangle\right),$$

where $\left|K_1^0\right\rangle$ $\left|K_2^0\right\rangle$ are the weak eigenstates, and $\left|K^o\right\rangle$ $\left|\overline{K}^o\right\rangle$ the strong eigenstates. Furthermore, CP changes $\left|K^o\right\rangle$ into $\left|\overline{K}^o\right\rangle$ and vice versa. So, if we operate with CP on $\left|K_1^0\right\rangle$ we obtain an eigenvalue of +1, which is CP even. Similarly, if we operate with CP on $\left|K_2^0\right\rangle$ we obtain an eigenvalue of –1, which is CP odd. If CP is conserved, the weak eigenstates K_1 and K_2 above are identically the CP even and CP odd states, respectively

Because the K_1 and the K_2 are distinct under CP, their decay products must also be distinct. The K_1 decays primarily into a state with two pi mesons (pions), which is even under CP, while the K_2 decays primarily into a state with three pi mesons, which is odd under CP. The three pion state has a mass very close to the kaon. Because of this, there is very little extra energy, or phase space, available for the decay to occur. This causes the K_2 to decay much less readily than the K_1, which has significantly more phase space. In fact, the K_2 has a lifetime approximately 600 times greater than the K_1. For this reason the K_1 is usually called K_S and the K_2 is called K_L, where S and L designate the short and long lifetimes.

As mentioned, if CP is conserved, the K_S should be equivalent to the K_1 state and K_L should be equivalent to the K_2 state. Furthermore, if CP is conserved we expect that the K_1 (or K_S) should decay *only* into states such as $K_S \rightarrow 2\pi$, and the K_2 (or K_L) should decay only into states such as $K_L \rightarrow 3\pi$.

Cronin-Fitch experiment and Indirect CP violation

An obvious method to determine if CP is conserved is to produce a well-defined CP state and observe its decays. If the initial state has different CP than the final state, then CP has been violated. In 1964 Cronin et al. performed this type of experiment (Christenson et al., 1964). They produced a beam of K mesons, which was allowed to travel a relatively long distance. The region near the end of the beam line contained mostly K_2 mesons, K_1 mesons having decayed away, so they expected to only see decays from $K_2 \rightarrow 3\pi$. A signal for CP violation would be the appearance of $K_2 \rightarrow 2\pi$ decays in their detector.

The group used the Brookhaven AGS (Alternating Gradient Synchrotron) particle accelerator. A beam of protons bombarded a Be target producing an assortment of particles. Large magnets called *sweeping magnets* were used to remove charged particles so that a neutral kaon beam was produced. As the kaons proceeded down the beam line, they decayed, and the resulting daughter particles were detected by two detectors located at the end of the beam line. These detectors measured the momentum and positions of the daughter particles, which could be used to determine the momentum vector of the parent kaon particle. Because the detector only reconstructed two of the three daughter particles, the momentum vector of 3π decays

reconstructs to a non-zero angle with respect to the original K_2 beam. On the other hand, the momentum vector of 2π decays reconstructs coincident with the original K_2 beam. A sophisticated computer program was used to determine the expected results of this angle if CP were conserved. This *Monte Carlo simulation* was compared to the results recorded from the experiment. Figure 1 shows the distribution of the angles between the reconstructed momentum vector and original K_2 beam for all decays in a given mass region. Notice the significant peak above the Monte Carlo expectation, corresponding to zero angle decays. After subtracting backgrounds the group measured 45 ± 10 $K_2 \rightarrow \pi^+ + \pi^-$ decays of the 22,700 K_2 decays – the first evidence of CP violation.

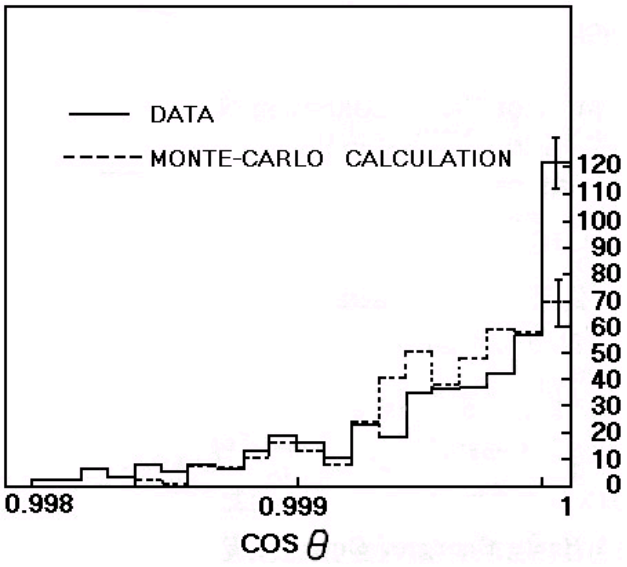

Figure 1. Angular distribution of events with mass 490 < m* < 510 MeV

Indirect CP violation

The Cronin experiment demonstrated that CP is violated in weak decays. This discovery lead to the realization that the weak eigenstates are not the same as the CP eigenstates. In fact, one can write the weak eigenstates as admixtures of the CP eigenstates

$$\left|K_S^0\right\rangle \propto \left|K_1^0\right\rangle + \varepsilon\left|K_2^0\right\rangle$$
$$\left|K_L^0\right\rangle \propto \left|K_2^0\right\rangle + \varepsilon\left|K_1^0\right\rangle .$$

In order for K_L and K_s to be pure CP eigenstates the mixing parameter must go to zero, conserving CP. However, the Cronin experiment demonstrates that e must be nonzero, so the weak eigenstates are not pure eigenstates of CP but contain a small amount of the "wrong" CP value. For example, K_S is not purely CP even but contains a small amount, e, of CP odd. Furthermore, we notice that e is independent of decay channel so we expect CP violating effects to have the same rate whether the decay mode is $K_L \rightarrow \pi^o + \pi^o$ or $K_L \rightarrow \pi^+ + \pi^-$. Because this violation is due to mixed eigenstates, it is referred to as CP violations through mixing or *indirect CP violation*. The Cronin experiment obtained a parameter value of $\varepsilon \sim 2.3 \times 10^{-3}$ which corresponds to 1 violation for every 500 decays.

Direct CP violation, NA48, and KTeV

Direct CP violation

The Cronin experiment showed that CP violation occurs when a state is composed of mixed CP eigenstates. The $K \rightarrow \pi\pi$ decay occurs through the K_1 component. CP violation however, might occur in the direct decay of either K_1 or K_2, so that the K_2 component might simply decay into two pions. This is called *direct CP violation*, the degree of which is measured by a parameter ε'. Note that this violation does not appeal to an admixture of CP states but occurs directly in the decay. This is why direct CP violations are sometimes referred to as *violations in the decay*. So there are two means by which CP may be violated. Either the K_1 component of the K_L decays into two pions (indirect CP violation). Or, the K_L decays into two pions via the K_2 state (direct CP violation). These possibilities are shown below:

$$\left|K_L^0\right\rangle = \underline{\left|K_2^0\right\rangle} + \varepsilon\underline{\left|K_1^0\right\rangle}$$

$\pi\pi$ indirect

$\pi\pi$ direct

ε'/ε and the Double Ratio

Although indirect CP violation manifests itself in the $K_L \rightarrow 2\pi^o$ decay, CP violation also occurs in other decay modes such as $K_L \rightarrow \pi^+ + \pi^-$. If all CP violating effects were due to indirect CP violation, which is decay channel independent, then the rates for different decay modes would be the same. So if one were to compare the indirect violation in two different modes, and find different rates, this must be due to a direct CP violation.

One method to search for direct CP violation is to compare $K \rightarrow \pi^0\pi^0$ decays to $K \rightarrow \pi^+\pi^-$ decays. The ratio ε'/ε can be determined from the following ratio of CP violating rates:

$$R = \frac{\dfrac{\Gamma\left(K_L \to \pi^+\pi^-\right)}{\Gamma\left(K_S \to \pi^+\pi^-\right)}}{\dfrac{\Gamma\left(K_L \to \pi^o\pi^o\right)}{\Gamma\left(K_S \to \pi^o\pi^o\right)}} \cong 1 + 6\,\mathrm{Re}\left(\frac{\varepsilon'}{\varepsilon}\right)$$

where Γ designates the rate of occurrence of the decay (Alavi-Harati, 1999). Notice that the numerator and denominator of this ratio are both measures of indirect CP violation, where we expect the K_L violations to occur about once in every 500 decays. If direct CP violation does not exist, we would expect the double ratio to equal unity, forcing the ε'/ε term to vanish as expected. However, if CP violations are decay channel dependent, the ratio *R* will deviate from unity resulting in a nonzero ε'/ε, evidence of direct CP violation.

NA48 and KTeV

Searches for direct CP violation have been performed since the Cronin experiment, but it was not until the last decade that experiments have obtained statistically significant results of direct violation. The difficulty in determining ε'/ε is due to the fact that direct CP violation is expected to be much smaller than indirect CP violation. About five years ago two promising experiments set out with a goal of obtaining a definitive value of ε'/ε.

The determination of the double ratio amounts to essentially a counting experiment. The two CP violating rates are just proportional to the number of particles from each decay mode within a certain decay volume. The difficulty in this measurement lies in efficiently determining the decay rates while subtracting errors from backgrounds and accidentals. One advantage of this measurement is that a number of systematic effects will cancel in the double ratio. So, it is important to collect all four decay modes simultaneously. As mentioned, the Cronin experiment recorded about 22,000 events of which 45 ± 9 were indirect CP violating. However, to measure ε'/ε to the desired precision nearly 20 million indirect CP violating decays have to be collected (Alavi-Harati, 1999).

In 1997, a group in experiment NA48 at CERN took data used to analyze ε'/ε. In three separate runs that lasted months at a time, NA48 was able to record over ten million events per run. This large number of events is possible due to the high rates produced by today's particle accelerators and the high efficiency of modern particle detectors. NA48 utilized a liquid krypton calorimeter to measure energy deposition and a bent crystal to extract protons onto a K_S target. Such a setup is necessary to produce a simultaneous collinear K_S beam, which is required to eliminate errors in the double ratio. After three years of taking data, NA48 reported a value of (15.3 ± 2.6) X10^{-4} for ε'/ε (Lai, 2001).

A second group under the KTeV collaboration used the Tevatron at Fermilab in investigation of ε'/ε. The setup and general methods are similar with NA48, but various components are constructed differently. For example, KTeV uses a pure CsI crystal calorimeter to measure energy deposition. To create the collinear K_S beam, KTeV uses a unique property of kaons whereby a K_L turns into a K_S when passed through a thin slab of matter. This is due to the strong interactions of kaons with protons and neutrons when passing through matter. (This *regeneration* process was also studied in the Cronin experiment.) Every second, 200 billion protons hit a beryllium target similar to the one used in the Cronin experiment (Cheu, 1997). An extremely high rate *trigger* determines 53 million times a second which events to use in data analysis. This corresponds to 10 MB of data every second, which over the KTeV running period, results on the order of 100 TB of data collected. After analyzing only one eighth of their data, the KTeV group was able to attain a statistically significant result much larger than the theoretical expectations of (4-10) X 10^{-4} (Ellis, 1976 and Ciuchini,1997). Combined with later data, which amounts to about one-half of the total KTeV data, resulted in a value for Re (ε'/ε) of (20.7 ± 2.8) X 10^{-4}.

Overall Average

After averaging all recent direct CP violation experiments, one obtains a world average of (17.2 ± 1.8) X 10^{-4} as shown in Figure 2. This result conclusively shows that direct CP violation exists, a new form of matter/antimatter asymmetry. Note that the small value of this result signifies that direct CP violation is much smaller than indirect violation.

The Future of CP Violation

KOPIO

The KTeV and NA48 experiments combined with the Cronin experiment definitively show that there are two types

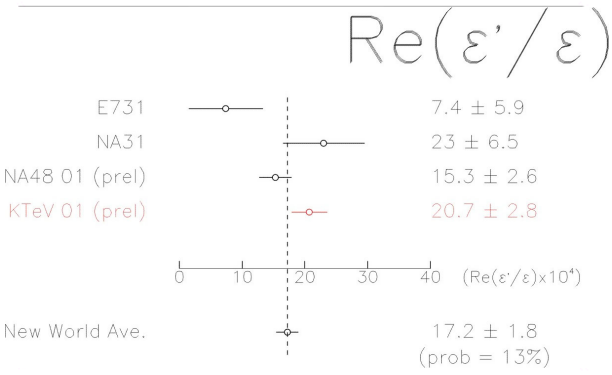

Figure 2. Values reported by various experiments for ε'/ε and the world average.

of CP violation: direct and indirect. However, theoretical calculations of ε'/ε are very challenging because of our lack of understanding of the strong force. To achieve a better understanding of CP violation, an investigation of other decay channels is necessary. The KOPIO experiment using the Brookhaven AGS – the same used by Cronin et al. – will attempt to study one of the more rare decay channels. In particular, the decay $K_L \rightarrow \pi^o \nu\bar{\nu}$ has some interesting features which are worthy of investigation. In this decay the amplitude for the indirect CP violating component is actually expected to be much less than the amplitude for the direct component. In addition, the theoretical uncertainties for this decay mode are very small because the final state does not include any hadronic particles such as pions, which are theoretically difficult to handle because they involve the strong interaction. So, measurement of this decay mode would allow a much better understanding of direct CP violation (Littenberg, 1989). This decay occurs about once in every 100 billion decays, making it particularly difficult to study. Experimentally, the final state consisting of two photons (from the π^o) and two neutrinos is difficult to reconstruct. Because the neutrinos only interact via the weak interaction, they will remain undetected in the experimental apparatus, so the challenge is locating two isolated photons and nothing else in the detector. Given the very high rate of backgrounds expected in this experiment, the KOPIO group faces a number of challenging tests in searching for CP violation in rare decays. However, a positive result from this group will help to further our understanding of CP violating effects.

New Detectors

The next generation of particle detectors and the next batch of CP violation experiments will continue to push the limits of our current technology. More than likely, the next generation of experiments will require much more precise and accurate detectors with the ability to obtain high precision under extreme conditions. One such detector has been examined under the KTeV project at the University of Arizona.

This detector is composed of alternating bars of quartz and uranium, and is situated directly in the beam line. This is a unique region for detectors, which are usually adjacent to the beam line where the interaction rate is much lower. A detector residing in the beam line is well-suited for experiments like KOPIO, where there are many background processes to a decay such as $K_L \rightarrow \pi^o \nu\bar{\nu}$. Such backgrounds can fake a signal if some of the daughter particles continue down the beam line and remain undetected. Placing such a detector in the beam line will reduce the probability for such backgrounds. The quartz-uranium detector operates by measuring the scintillation light resulting from interactions of charged particles with the quartz bars. Photons also can be detected as they shower in the heavier uranium, producing a multitude of charged and neutral particles. The measure of the resulting scintillation light is proportional to the amount of energy deposited.

In analyzing the detector's efficiency, K_L decays were reconstructed in the KTeV detector. Since the environment in the KTeV detector is similar to that expected in future experiments, this analysis should indicate how our quartz-uranium detector will perform in an experiment such as KOPIO. The electrons from K_L decays are identified using the KTeV detector, which is upstream of our quartz-uranium detector. Then, with the particles' identified, we test the ability of the quartz-uranium detector to correctly identify them. Despite the high rate environment of over 1 MHz, the detector is still able to properly identify particles correctly with very high efficiency (in this case electrons). In the KOPIO experiment, one is more interested in photons, but since electron and photon interactions are expected to be similar in our detector, these results indicate how well our quartz-uranium detector should perform for photons. Our result is no misidentified electrons from more than 40,000 events used in analysis. This places an upper limit of the detector's inefficiency at 5.0×10^{-5} at the 90% confidence level. This high efficiency signifies a promising future for tomorrow's detectors in future experiments such as KOPIO.

Conclusions

CP Violation Today

Although indirect CP violation was first discovered by Cronin et al. in 1964, the search for direct CP violation is a fairly recent endeavor. It was only in the last decade that a high enough precision has been reached to verify direct CP violation. Direct CP violation has been discovered and, as expected, it has a much smaller effect than indirect violation. Furthermore, CP violation is still in the early stages of understanding. We await experiments such as KOPIO to obtain a new outlook on direct CP violation. An interesting possibility in these experiments is the discovery of physics beyond the standard model. The standard model is the set of current theoretical facts, confirmed from experiment, which govern particle physics. The standard model is able to make certain theoretical predictions which experiment will confirm or deny. Thus far the standard model is consistent with experiment; however, if an inconsistency is discovered this will give rise to go beyond current

physics and establish a new theory. CP violation is a phenomenon which may provide this avenue away from the standard model.

Baryogenesis Revisited

Over the past three decades a preference of $\overline{K}^o \rightarrow K$ over $K \rightarrow \overline{K}^o$ has been established through CP violation. Thus one of the Sakarov conditions is now known to exist. Note however, that although CP violation exists, CP violation in weak decays cannot account for the degree of matter/antimatter asymmetry in the Universe. In fact CP violation in weak decays is too small by a large factor to account for baryogenesis. Future experiments will search for CP violation elsewhere, which is believed to manifest itself B meson system also; however current theory points to no resolution even under this consideration. To resolve this issue we might need to appeal to CP violation in models currently beyond the standard model and outside of the realm of current experimental confirmation. It may be that only under considerations of Supersymmetric models (SUSY) or models at the level of Grand Unified Theories (GUT) will the riddle of matter dominance finally be solved.

About the Author

James Morris is an undergraduate senior attending the University of Arizona. He plans on attending a doctorate program in physics. He aspires to become a professor of physics at the university level and to contribute to current research in the field. James assists the high energy department at the University of Arizona. In particular he is testing the efficiency of next-generation particle detectors for KTeV which may be used in future experiments involving CP violation. The detector under analysis is a quartz-uranium detector which has the uncommon feature of residing directly in the beam line. Such a region is rare for particle detectors, which usually reside adjacent to the beam line in detecting electromagnetic showers. This region exposes the detector to a high rate environment on the order of 1 MHz. And this fact combined with the higher precision required by future experiments creates the need for a high efficiency. Despite these circumstances the detector is still able to attain high efficiency.

Acknowledgements

I would like to thank the High Energy group at the University of Arizona for their assistance and use of their resources. I am particularly grateful to Dr. Elliott Cheu for his assistance and guidance on this project.

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Reviews in Undergraduate Research, Vol. 1, 44-50 , 2002

The Role of Caveolae and the Caveolins in Mammalian Physiology

Dr. Babak Razani[1] and Dr. Michael Lisanti[1]

Caveolae are 50-100 nm invaginations of the plasma membrane that have captured the interest of scientists for many decades. However, the wide-ranging and physiologically important roles of these curious structures have only recently been addressed. Among the important milestones in the understanding of caveolae is the discovery of a family of proteins that are intimately involved in caveolar function (the caveolins). It has become clear now that caveolae and their caveolin "marker proteins" are involved in a variety of cellular processes including endocytosis, lipid homeostasis, signal transduction, and tumorigenesis. In this review, we will highlight the current view of caveolae in cell biology and discuss the relevance of these structures to mammalian physiology.

Introduction to Caveolae

Examination of a cell at the ultrastructural level reveals numerous intricate components that contribute to its appropriate function. Since the advent of electron microscopy in the 1940s and 50s, structures such as the mitochondrion, endoplasmic reticulum, golgi apparatus, and clathrin-coated endocytic vesicles were discovered for the first time and their distinct functions in cells speculated upon. In this same period, another cellular entity, a 50-100 nm vesicle that was found to be either directly invaginated from or in close proximity to the plasma membrane was also described (Figure 1A). Based on this conspicuous "cave-like" morphology at the membrane, these structures were named "*caveolae*" and were added to the growing list of newly discovered cellular organelles (Palade, 1953; Yamada, 1955).

In the ensuing decades and with the incipience of cellular and molecular biology, research on many of these cellular organelles led to a precise understanding of their function (e.g. implication of mitochondria in ATP production, the ER/golgi in protein synthesis and sorting, and clathrin-coated pits in endocytosis).

Unfortunately, due to difficulty in characterizing their biochemical and molecular nature, caveolae remained enigmatic structures with no definitive function(s). Based on their structural resemblance to clathrin-coated vesicles and their seemingly dynamic movement between the plasma membrane and intracellular compartments, caveolae were initially thought to serve solely an endocytic role akin to clathrin-coated pits (Palade, 1953; Simionescu et al., 1975).

Now, based on work in the last decade, caveolae are being recognized as rather complex organelles with important roles not only in endocytosis but also lipid homeostasis, signal transduction, and tumorigenesis. In addition, they seem to play very specific roles in distinct cell types, making these structures one of the most interesting and multi-functional entities in cells. In this review, we will discuss the salient features of these structures and the current understanding of their function in mammalian organisms.

The Biochemical/Structural Nature of Caveolae: Introduction to the Caveolins

Based on numerous biophysical and biochemical analyses of plasma membranes, it is now known that the traditional view of a lipid bilayer as a "fluid mosaic" is not entirely accurate (Brown and London, 1998). Although a membrane solely made of phospholipids does indeed act as a fluid-mosaic, cell membranes which are also composed of cholesterol, sphingolipids, and various lipid-modified and transmembrane proteins, behave differently (Brown and London, 1998). In cell membranes, depending on the local concentration of cholesterol, sphingolipids, and some phospholipids, more rigid patches of membrane can form. Floating among the bulk phospholipids bilayer, these biochemically distinct patches of membrane have now been termed lipid rafts, the study of which is an active area of research (see (Simons and Toomre, 2000) for review) (Figure 1B).

Interestingly, research in the past decade has shown that caveolae are biochemically indistinguishable from lipid

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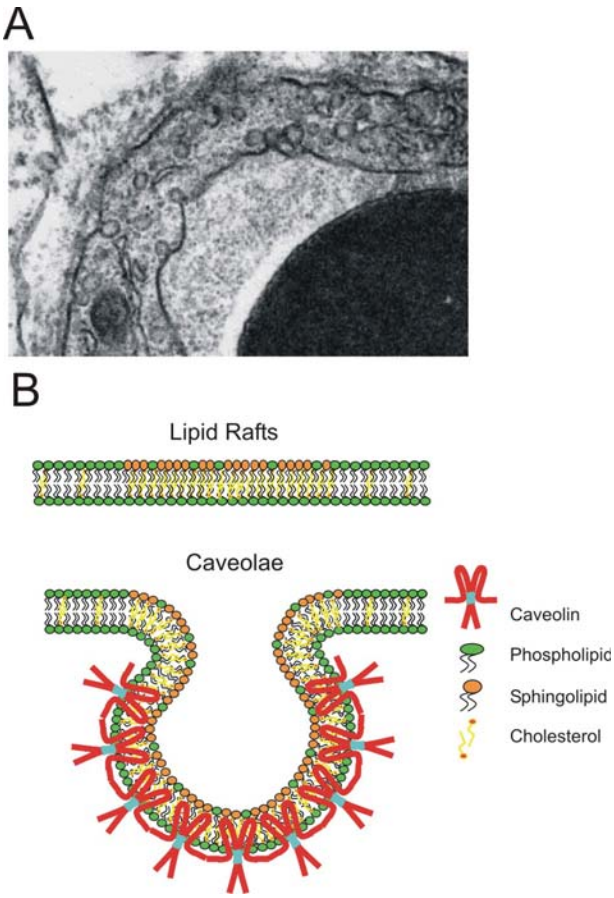

Fig. 1. Figure 1. Caveolae, a unique "cellular organelle" with a unique "marker protein"

A) Electron micrograph of an endothelial cell showing caveolae, 50-100 nm structures that are either direct invaginations or in close proximity to the plasma membrane. Caveolae are estimated to make-up an estimated 30-70% of the plasma membrane area in certain cells such as endothelial cells, adipocytes, or Type I pneumocytes .

B) Diagram comparing the biochemical composition of lipid rafts and caveolae (adapted from (Galbiati et al., 2001)). Lipid rafts form via a coalescence of cholesterol and sphingolipids; as a result, these microdomains have vastly different biochemical properties than the bulk phospholipids bilayer. Caveolae are generally considered to be "invaginated" lipid rafts primarily due to an enrichment in a family or proteins known as the caveolins. Here, the caveolin oligomer is depicted as a dimer for simplicity.

rafts and are composed of a similar local enrichment of cholesterol and sphingolipids (Simons and Toomre, 2000) (Figure 1B). The primary difference between these two entities is the invaginated, vesicular morphology of caveolae. This difference arises due to the presence of a set of proteins unique to caveolae but absent from lipid rafts, the caveolins.

The caveolin protein family is composed of three distinct proteins, caveolin-1, -2, and -3 (Cav-1, -2,-3) (Glenney, 1992; Rothberg et al., 1992; Scherer et al., 1996; Tang et al., 1996). Not surprisingly, these proteins are expressed in tissues with a high abundance in caveolae; Cav-1 and -2 are co-expressed in many cell types with especially high levels in endothelial cells, adipocytes, and type I pneumocytes, while Cav-3 is exclusively expressed in skeletal and cardiac muscle cells (Scherer et al., 1994; Scherer et al., 1996; Tang et al., 1996). The main exception is smooth muscle cells, where intriguingly all three proteins are expressed (Tang et al., 1996).

The caveolin proteins have several properties which are important not only for selective localization to caveolae but also for driving the invagination of these structures. Cav-1 has been shown to have high binding affinity for cholesterol and sphingolipids (Fra et al., 1995; Murata et al., 1995; Thiele et al., 2000). This property, along with three carboxy-terminal lipid-modifications (palmitoylations), stabilizes and targets Cav-1 to caveolae (Dietzen et al., 1995; Monier et al., 1996). The caveolins can also oligomerize into a complex of 14-16 subunits and thereafter form even larger mega-complexes by oligomer-oligomer interactions (Monier et al., 1995; Sargiacomo et al., 1995; Song et al., 1997). Although it is still largely speculative, it is thought that the high affinity for cholesterol, the oligomerization, and the oligomer-oligomer interactions can together form an environment in the lipid bilayer conducive for the creation of 50-100 nm caveolar invaginations.

Caveolae, Caveolins, and Endocytic Processes

The observation that caveolae can exist as invaginations of the plasma membrane, as completely enclosed vesicles, or as aggregates of several vesicles, led investigators to infer that these structures were conduits for the endocytosis of macromolecules (Simionescu et al., 1975). Indeed, tracer studies and high resolution electron microscopy has revealed that cells predominantly use caveolae for the selective uptake of molecules as small as folate to full size proteins such as albumin and alkaline phosphatase (Anderson et al., 1992; Parton et al., 1994; Predescu et al., 1997; Schnitzer et al., 1994) (Figure 2A). In endothelial cells, the uptake of such molecules is complicated by the fact that the endocytosed caveolae seem to migrate from the luminal side to the abluminal side, thereby transferring specific serum molecules to the underlying tissue (a process referred to as transcytosis) (Predescu et al., 1997; Simionescu et al., 1975) (Figure 2A).

Interestingly, several studies have also shown that caveolae-mediated uptake of materials is not limited to macromolecules; in certain cell-types, viruses (e.g. simian virus 40) and even entire bacteria (e.g. specific strains of E. Coli) are engulfed and transferred to intracellular compartments in a caveolae-dependant fashion (Anderson et al., 1996; Montesano et al., 1982; Shin et al., 2000).

Although the molecular mechanism for these endocytic events are not completely understood, there are indications that the same machinery operating traditional vesicle budding and fusion processes is functional in this setting (Henley et al., 1998; Oh et al., 1998; Schnitzer et al., 1995). Thus, the cell utilizes similar endocytic techniques to differentially traffic cellular materials.

Caveolae, Caveolins, and Cholesterol Homeostasis

Caveolae are highly enriched in cholesterol as compared to the bulk plasma membrane and Cav-1 binds this cholesterol with high affinity (estimated at 1 cholesterol molecule per caveolin molecule) (Murata et al., 1995; Thiele et al., 2000). Furthermore, pharmacological depletion of plasma membrane cholesterol leads to a loss of morphologically identifiable caveolae (i.e. "flattening" against the membrane) and dissipation of the caveolin-matrix (Rothberg et al., 1992). Due to these observations, it was suggested that caveolae and caveolins are involved in maintaining intracellular cholesterol balance; indeed, there is evidence for such a role.

Cellular cholesterol is derived from two main sources, de novo production or extracellular uptake (via low density lipoprotein (LDL) receptors localized in clathrin-coated vesicles) (Fielding and Fielding, 1997; Simons and Ikonen, 2000) (Figure 2B). Once inside, the caveolins seem to function as intracellular escorts for the transport of this cholesterol from the endoplasmic reticulum to plasma membrane caveolae (Smart et al., 1996; Uittenbogaard et al., 1998) (Figure 2B). Upon delivery, this cholesterol has three fates: (1) to remain as a component of caveolar cholesterol, aiding in the invagination and proper function of these structures, (2) to be siphoned into the bulk plasma membrane, repleting the lipid bilayer with appropriate amounts of cholesterol, or (3) to be effluxed to serum cholesterol-transporting units like high density lipoproteins (HDLs) (Fielding et al., 1999; Fielding and Fielding, 1995; Smart et al., 1996) (Figure 2B). In essence, the caveolins deliver intracellular cholesterol to a "relay station" wherein the overall fate of cholesterol is determined; the cholesterol needs of the cell are met and excesses are effluxed.

Caveolae, Caveolins, and Signal Transduction

The intimate relationship between caveolae and their protein components, the caveolins, is obvious. An important question that remained was whether other plasma membrane proteins can also preferentially localize to these structures. This issue has been addressed using biochemical purification, wherein caveolae can be selectively isolated from other cellular constituents and their protein components analyzed (Lisanti et al., 1994; Sargiacomo et al., 1993).

Caveolae are highly enriched in numerous membrane-bound proteins, especially signaling proteins with lipid-modified groups (e.g. H-ras, src-family tyrosine kinases, heterotrimeric G-proteins, eNOS, etc) (Lisanti et al., 1994; Smart et al., 1999) (Figure 2C). Furthermore, it appears that the caveolins are not innocent by-standers in this environment and can bind and functionally regulate (mostly inhibit) several of these caveolae-localized molecules (Feron et al., 1996; Garcia-Cardena et al., 1996; Li et al., 1996; Li et al., 1995; Song et al., 1996; Song et al., 1997) (Figure 2C). The caveolins possess a 20 amino acid juxtamembrane domain (now appropriately called the scaffolding domain) that mediates this functional binding (Okamoto et al., 1998).

The predilection for signaling proteins to localize to caveolae and the capacity for the caveolins to regulate their function has led some to refer to caveolae as "signalosomes" (or bodies where signal transduction events and cross-talk between different signaling pathways can take place efficiently and in regulated fashion) (Lisanti et al., 1994; Smart et al., 1999). This aspect of caveolae is currently an active area of research since it brings together the interests of investigators conducting research in seemingly disparate areas.

Caveolae, Caveolins, and Tumorigenesis

An interesting corollary to the above-mentioned signalosome concept arises during tumorigenesis. Several of the proteins localized to caveolae and inhibited by Cav-1 (namely EGFR, Her2/Neu, and PDGF receptor tyrosine kinases, components of the Ras/p42/44 MAP kinase cascade, and members of the PI-3-kinase cascade)) (Couet et al., 1997; Engelman et al., 1998; Liu et al., 1996; Yamamoto et al., 1999; Zundel et al., 2000) are extremely important in pro-proliferative/anti-apoptotic signaling. If functionally deranged, such proteins can result in cells with hyperactive cell cycles and eventually tumor formation. In this regard, caveolae and Cav-1 might be expected to be essential members of the cellular tumor suppressor repertoire, acting to dampen the action of tumorigenic signals.

Interestingly, it has been observed that caveolae are absent or reduced in number and Cav-1 is transcriptionally down-regulated in numerous cancers (both cell-lines and in situ carcinomas) (Engelman et al., 1998; Koleske et al., 1995; Lee et al., 1998; Razani et al., 2000). In addition, both human CAV-1 and -2 genes map to 7q31.1 (a region of the chromosome found to be frequently deleted in several epithelial cancers - e.g. breast, lung, renal, and ovary) (Kerr et al., 1996; Shridhar et al., 1997; Zenklusen et al., 1994). Such observations provide strong evidence for a caveolin-mediated tumor surveillance process and give impetus for researchers to include the caveolins as important factors in the diagnosis and treatment of cancer.

In vivo Relevance of Caveolar Function in Mammalian Physiology

The current understanding of caveolae and caveolin function is based on research conducted either in vitro (biochemical or cell culture systems) or in vivo (namely, morphological assessment by electron microscopy). Although such techniques are useful in providing insights into the functions of these structures, a complete understanding of their physiological relevance can only be attained by experiments conducted in the whole organism (e.g. creation of transgenic or knockout mice, wherein the expression of one or more caveolin proteins is perturbed).

Indeed, in the past year, several groups have reported on the phenotypes of mice with targeted disruptions of the CAV-1, -2, and -3 loci, thereby providing the first rigorous assessment of caveolae function in vivo (Drab et al., 2001; Galbiati et al., 2001; Hagiwara et al., 2000; Razani et al., 2002; Razani et al., 2001; Razani et al., 2002). Mice deficient in Cav-1 or Cav-3 (but not Cav-2) lack morphologically identifiable caveolae in tissues expressing those genes. This observation is important in that it directly proves the importance of caveolin expression in caveolae formation and provides a tool for the study of not only caveolins but caveolae in a mammalian organism.

The phenotypes of Cav-1 null mice (Drab et al., 2001; Razani et al., 2002; Razani et al., 2001):

1) Loss of caveolae in cells expressing Cav-1 (e.g. endothelial, epithelial, adipose cells)
2) Dramatic reduction of Cav-2 protein levels due to destabilization and degradation via the proteosomal pathway - thus, Cav-1 null mice are in essence Cav-1 and -2 deficient
3) Histologically abnormal lungs - thickened alveolar septa due to endothelial cell hyper-proliferation and increased

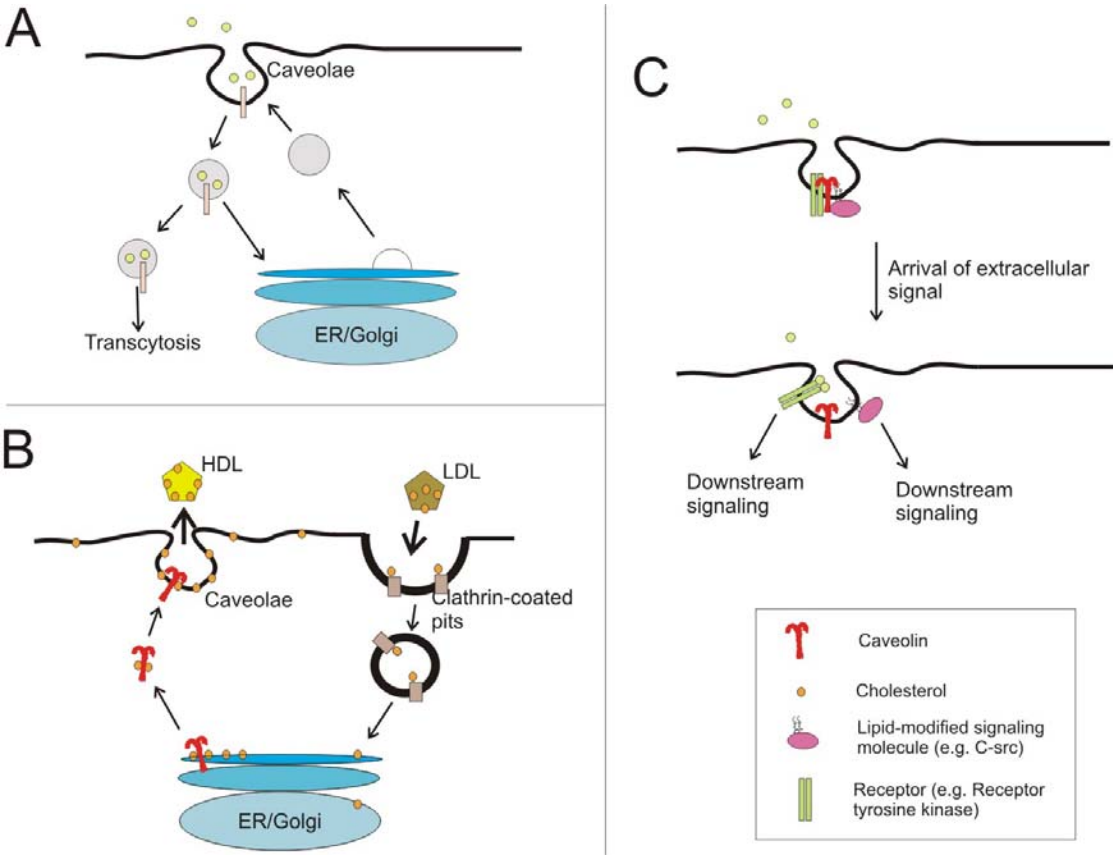

Figure 2. Proposed functions of caveolae and the caveolins (adapted from (Razani and Lisanti, 2001))
A) Certain molecules have been shown to be predominantly endocytosed via caveolae and not clathrin-coated vesicles. The fate the cargo in a fully invaginated caveola is not entirely understood; however, there is evidence to suggest that depending on the cell type, caveolae can deliver their contents to the ER/golgi compartments or to the abluminal side of a cell.
B) Intracellular cholesterol is thought to be transported to plasma membrane caveolae via a golgi-independent caveolin-mediated route. Caveolae then can serve as "relay stations" to deliver the membrane cholesterol to the bulk plasma membrane or to cholesterol-transporters such as HDL particles.
C) Caveolae are now thought to act as signalosomes, or entities in which signal transduction events can take place efficiently. A higher level of regulatory complexity is provided by the caveolins where signaling molecules can be bound until extracellular ligands relieve them of inhibition. Here, the dynamic regulation of a receptor tyrosine kinase (e.g. EGF receptor) and a lipid-modified kinase (e.g. the src-tyrosine kinase) in caveolae are shown.

deposition of extracellular matrix
4) Cell cycle defects - fibroblasts derived from these mice have increased S-phase fractions and proliferate faster than their wild-type counterparts

5) Defects in vasoregulation - the aortas from these mice are hyper-responsive to vasodilatory stimuli due to hyper-activation of the eNOS signaling cascade
6) Defects in endocytosis - the uptake of albumin by endothelial cells is drastically reduced in these mice
7) Defects in lipid homeostasis - these mice are resistant to diet-induced obesity and have histological abnormal adiposities with age. These mice are also hypertriglyceridemic with a reduced capacity to clear serum lipids, a condition likely related to the aberrant adipose function.

The phenotypes of Cav-2 null mice (Razani et al., 2002):

1) Normal or slightly reduced Cav-1 expression with no loss of caveolae - thus, these mice are extremely useful for comparison with Cav-1 deficient mice in that they selectively lack Cav-2
2) Histologically abnormal lungs - in fact, the lung defects in these mice are indistinguishable from Cav-1 null mice, thereby for the first time demonstrating an important role for Cav-2 independent of Cav-1
3) Unperturbed vasoregulation and lipid homeostasis - these observations were important in establishing that Cav-1 and Cav-2 have distinct and non-overlapping roles in physiology.

The phenotypes of Cav-3 null mice (Galbiati et al., 2001; Hagiwara et al., 2000):

1) Loss of caveolae in cells selectively expressing Cav-3 (i.e. skeletal and cardiac muscle)
2) Histologically abnormal skeletal muscle with necrotic muscle fibers and centralized nuclei - indeed, this mild muscular dystrophy phenotype recapitulates the pathology seen in a previously described group of patients with Limb-girdle muscular dystrophy (type 1C) in which mutations in the CAV-3 gene were found (Minetti et al., 1998).
3) Defects in the myocyte T-tubule network with irregularly-oriented tubules

Conclusions and Future Directions

As can be seen from the above description, the initial characterization of these mice has provided a wealth of information ranging from the predicted (e.g. involvement in endocytic processes and signaling cascades such as eNOS) to the completely unexpected (e.g. lung hypercellularity and defects in triglyceride rather than cholesterol homeostasis).
The study of caveolae and their marker proteins, the caveolins, has been an exciting yet challenging endeavor. The ever-changing view of their function in mammalian physiology is in part due to the difficulty of working with such membrane domains and a lack of different but complementary tools available for rigorous analyses. Caveolae and the caveolins have thus far been implicated in endocytosis, lipid homeostasis, signal transduction, and tumorigenesis. Now, with the availability of caveolin-deficient mice, biochemical, cell culture, and genetic approaches can finally be intermeshed to provide a more complete picture of caveolar function in vivo.

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