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Review

Chicken-specific peptide arrays for kinome analysis: Flight for the flightless

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ABSTRACT

Kinomics, the study of kinase enzymes within an organism, is a rapidly growing field of proteomics. The use of high-throughput technology to study the kinome has enabled researchers to conduct studies of the global signaling environment within an organism. The problem arises when researchers interested in non-human, non-mouse, species attempt to use these latest techniques for their species of interest. A recent advancement which has overcome this species problem is the species-specific peptide array. Custom tailored to the species of interest this high-throughput kinome technology allows researchers to study global cellular signaling events in nearly any organism that uses phosphorylation-mediated signal transduction. Specific to this review is the study of the chicken which has never been more important or relevant research species. There are a number of basic biological questions about chickens that can be answered through new experimental techniques. In addition, zoonotic diseases, like avian influenza and *Salmonella*, which can infect humans through interaction with infected animals, have shown avians to be an important infectious vector. While the significant limitations to the mouse model have become more and more apparent, researchers have turned to alternative species such as chicken which are relatively easy to care for, inexpensive and are suited to large scale studies. The chicken is an ideal candidate for *in ovo* developmental studies as well as models for certain infectious agents. Finally issues of food safety and agricultural antibiotic use are ever present in the media and public policy discussions. The development of research tools to find safer means of animal production and alternatives to antibiotics are going to be increasingly important research objectives in the years to come.

KEYWORDS: kinome, chicken, peptide array, phosphorylation, chicken model

Kinomics

The field of genetics has had a large influence on the life sciences over the past several decades. At the turn of the new millennium the study of whole genomes, genomics, was heralded as a new frontier of science and medicine. The human genomic project, which was undertaken to undercover the entire human DNA sequence was considered a short step away from determining the mechanism of nearly every human disease and condition [1]. While sequencing whole genomes is an important tool for scientific discovery it is also extremely complicated to translate that to host biology and phenotype. Developed in parallel with genomics was transcriptomics, the study of the transcriptionally active subset of the genome. Considering only the transcriptome significantly simplified the work of geneticists as it was thought to only represent a small fraction of the

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entire genome. After genomics, transcriptomics is one of the more mature "omic" disciplines and is widely applied [2]. A number of tools are available for the study of gene transcripts not least of which being quantitative polymerase chain reaction, cDNA microarrays and next-generation sequencing techniques. A significant drawback of considering only the transcription of genes is that one assumes a great deal as several subsequent processes have yet to take place. Gene transcription does not necessarily mean translation; mRNA can be silenced or destroyed before protein can be produced. In addition proper folding, post-translational modifications and activation are central to the proper functioning of a protein. When considering only the level of mRNA a significant amount of molecular biology is left out including, gene silencing, mRNA stability, translational efficiencies, protein turnover, sequestration of enzymes from substrates and the activation/deactivation tuning by a multitude of post-translation modifications, of which phosphorylation is a major class.

When considering the effects of a condition, treatment or disease on a cell, tissue or organism studying the protein level as opposed to the gene or transcript level reduces complicating variables. The proteome contains the final effectors resulting in the organism's phenotype. When considering enzymes it is only those that are functionally active that can exert their effect. For example a gene encoding a protein may be transcribed but not translated, having no effect on the cell. Similarly an enzymatic protein may be translated but due to sequestering of the protein within an organelle or a lack of an activating post-translational modification the enzyme may never exert an effect on its substrate, thus affecting the cell. Cellular signaling via phosphorylation plays a central role in the regulation of nearly every aspect of cellular behavior [3], considering the active kinase enzymes carrying out these phosphorylation events can provide insight into nearly every cellular function. The kinase subset of the genome or proteome is called the kinome and its study is referred to as kinomics. Protein phosphorylation can modulate protein confirmation and function and kinases control processes including metabolism, transcription, apoptosis, cell differentiation, cell cycle progression, cytoskeletal rearrangement and movement among others. Considering some of the potential limits to a genetic approach and the central importance of phosphorylation-mediated signal transduction, the study of kinomics has the potential to provide knowledge of cellular biology and cellular responses to stimuli that has thus far eluded researchers using other approaches.

Peptide arrays for kinomics

The use of peptide arrays is one of a handful of techniques commonly used to study the kinome. It has a number of advantages over other techniques such as antibody based arrays and mass spectrophotometry, including the wide availability of reagents and the ability to focus only on the active kinases within a sample. The basis of peptide arrays for kinome analysis is the use of peptides which represent kinase enzyme target sites, these peptides are synthesized and printed onto an array surface [4]. A sample containing active protein kinases, either a mixture of purified kinases or a cellular lysate, is applied to the array. The active kinases within the sample recognize their respective kinase target sequences and, using the γ-phosphate group from added adenosine triphosphate (ATP) as a donor, phosphorylate their respective serine, threonine or tyrosine residue on the target sequence. The phosphorylation event can be visualized by a number of methods including phosphorylation-specific antibodies [5], radioactivity [6, 7], a labeled chelator [8] or phospho-specific stains [9]. Quantification of phosphorylation of a given peptide sequence provides information on the active kinases within a sample as well as the phosphorylation state of the kinase targets within a cell under given conditions. The process outlined above is shown in Figure 1.

There are numerous advantages to using only the kinase target sequence as opposed to the complete protein sequence on the array. While considering the reaction between kinase and full protein substrate would provide similar information on phosphorylation state, synthesizing a full length protein is a much more significant process than a short peptide sequence and full length proteins are often unstable on an array format. Little is lost in

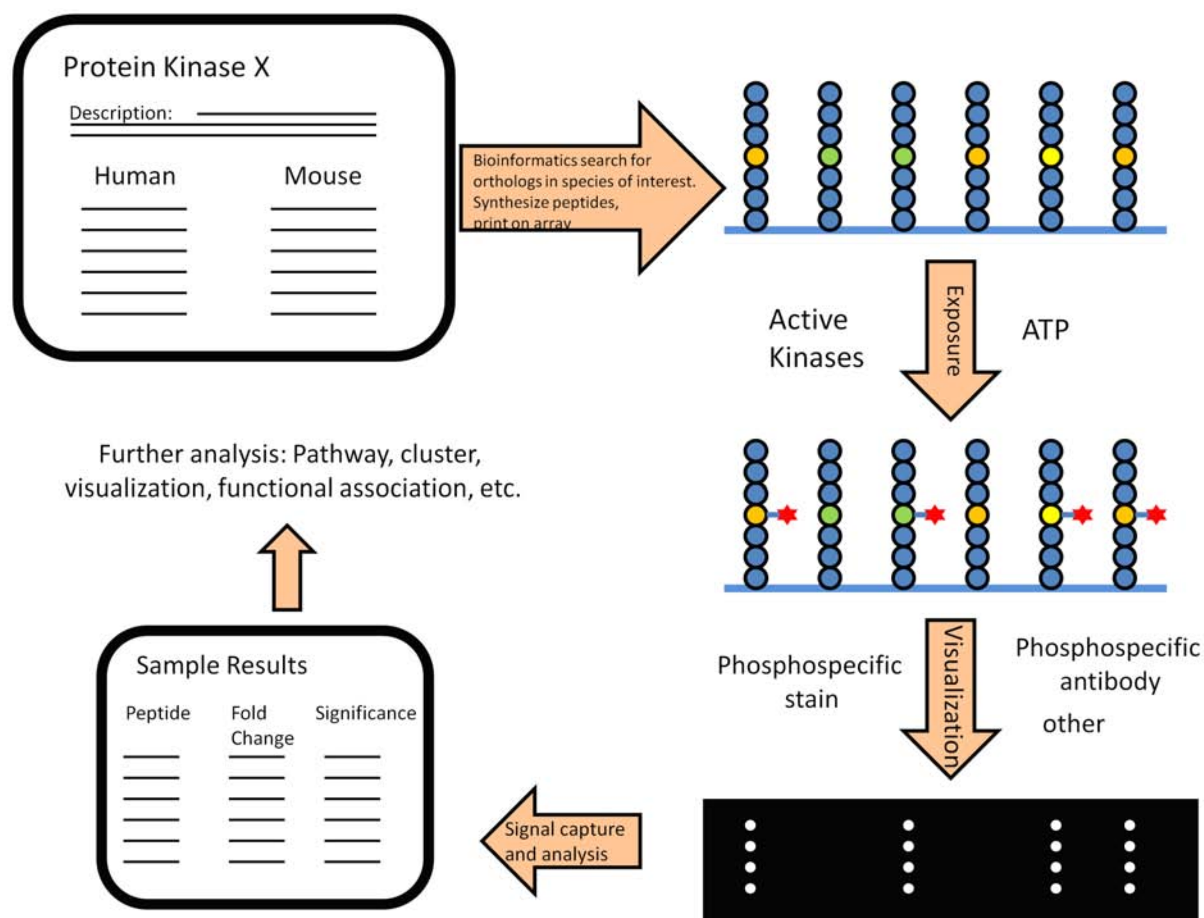

Figure 1. Kinome peptide array. Online databases of kinase target sites, predominantly human and mouse, are widely available. These can be used to find orthologous peptide targets in the species of interest, for example chicken. These peptides, usually 9-15 amino acids in length with a central serine, threonine or tyrosine residue, are synthesized and printed onto an array. The array is exposed to sample containing active kinases along with ATP as a phosphate group donor. The active kinases recognize and phosphorylate their respective target sequences on the array adding a phosphate group to the central residue. This phosphorylation event can be visualized by a variety of techniques. An array image is collected and the signal captured through the use of computer software. This signal is normalized and statistical analysis is applied to the data. The data can then be used to uncover biologically relevant information from the kinase sample which was exposed to the array.

taking a peptide approach since for many protein kinases the specificity of their substrate recognition is dictated by the amino acid residues immediately surrounding the phosphorylated residue rather than any higher-order structure. Specifically, the central phosphorylated amino acid plus the four flanking amino acids is the most common kinase recognition site [10]. Peptide used to represent the kinase target sequence has been shown to be an appropriate model for the kinetics of phosphorylation reactions, as well both V_{max} and K_m values are similar to those of full length protein [11]. One of the most significant advantages of peptides is the ability to investigate individual target sites; one can easily study multiple phosphorylation events on a single protein. It is a common feature of phosphorylated proteins to have multiple sites of phosphorylation, often affecting the behavior of the protein in different ways [12, 13]. For example a phosphorylation at one position on a protein may activate an enzymes catalytic activity, another site may deactivate the same enzyme and phosphorylation at a third site may cause the enzyme to be translocated to a

different location within the cell. Each of these phosphorylation events provides valuable information and each can be distinguished from the other by printing the distinct phosphorylation target sites for each on the array as separate peptides. The use of peptides as kinase targets is cost-effective, they are stable in an array format, provide site specific information, display similar biochemical properties to the full protein, and can be chosen to model nearly any protein which undergoes a characterized phosphorylation event [14].

Species-specific and chicken-specific kinome arrays

The first step in the design of a kinomic peptide array is the selection of the peptides which represent kinase phosphorylation target sites. It is estimated that up to one third of the human proteome undergoes phosphorylation representing at least 100,000 unique phosphorylation events [15]. The number of phosphorylation events and the complexity of the kinome can easily rival that of the genome considering the best estimate of the number of translated human genes is 22,333 [16]. Publically available databases of phosphorylation information such as PhosphoSitePlus (www.phosphosite.org) and Phospho.ELM (phospho.elm.eu.org) are invaluable sources of information on curated literature-based phosphorylation sites. The protein information provided by these databases include short peptide sequences corresponding to phosphorylation sites, as well as the functional changes brought about by a phosphorylation/ dephosphorylation at the given site. These databases have been used to design peptide arrays for kinome analysis [7]. Following the selection of the peptides of interest, peptide synthesis and array printing can be carried out either in-house or by a number of commercial companies.

These phosphorylation databases, as with most resources in this field, are biased toward human and mouse. This can be a distinct disadvantage when studying other species, especially a species like chicken. However, the large amount of information available in these databases can be used as a starting point for the design of species-specific peptide arrays tailored for chicken. Differences between human and chicken, human and mouse, or any two distinct species can be significant, especially when considering the proteome which appears to have more between-species variation than the genome [17]. However, the phosphorylation-mediated signal transduction pathways and the activities of the key constituents of these pathways are evolutionarily well preserved. As a result one can use the wealth of data available for the kinome of human and mouse to find orthologous sites in a species of interest, in this case chicken. This process was original performed and validated for bovine [18] and has subsequently been expanded for other species including chicken.

The advantage of this technique is that one is not relying on potential cross-reactivity between human phosphorylation target sites and chicken kinases. The target peptides are designed for the chicken so that the maximum interaction potential can be achieved when performing the analysis on chicken samples. It is clear from the work with antibodies that cross reactivity based on peptide epitopes of other species often do not interact with chicken protein. This design process avoids any of those issues.

The importance of chicken as a research topic

Chicken is a species well suited to scientific research. The relative abundance of animals and the low cost of procurement are two important considerations for researchers. In addition chickens are easy to care for and are suitable for large scale studies as they require limited housing space and relatively little food compared to some other agricultural species. This section outlines some of the reasons why chicken should be considered as an important species for scientific research.

Basic biology still unknown

Despite the length of time chicken has been a domesticated food production species and the extensive breeding of the species there are still a number of basic biological questions that have not been answered for the chicken, especially in the field of immunology. One of the unique aspects of the chicken immune system is the difference in the Toll-like receptors (TLRs) [19]. Chickens contain a number of distinct TLRs that are only found in avian species, these include TLR1LA, TLR1LB and TLR15. In addition, TLR21 in

chicken is only found elsewhere in fish and amphibians [19]. A better understanding of these receptors and the cellular signal transduction pathways they stimulate may provide answers to why the chicken immune system responds in a significantly different way to humans and also other avian species such as ducks and geese [20]. Another under studied aspect of the chicken immune system is the T regulatory (Treg) cells. In other species these cells are known to regulate the adaptive immune response, eliminating the potential for an autoimmune response [21]. Chickens appear to have a Treg system but as yet no specific markers for Treg cells have been found and chicken Tregs appear function similarly to mammals despite lacking the Treg differentiation factors found in mammals [22].

Even very basic intracellular biological functions in chickens are not well known. An example of this is the chicken response to insulin. It has been proposed that chickens do not respond to insulin, to the extent that chickens have been described as insulin resistant, however evidence to the contrary has been shown [23]. In addition key intermediates of the insulin signaling pathway were thought not to be present in the chicken proteome, thus eliminating the ability for chicken cells to respond to insulin, however these proteins has been found within the chicken genome and are thought to be expressed [24]. These questions of basic cellular biology are still open to discovery in the chicken and a comparative study with other well researched species may provide valuable information toward therapeutic interventions in these other species, including humans.

As models

A number of aspects of chicken development make it an ideal model for embryonic developmental studies, including the ease of experimental manipulation of the egg, a long track record of use in developmental biology, and the short developmental period [25]. The genetic diversity of the chicken due to breeding and natural selection make chicken the ideal species for genetic and evolutionary study [26].

One of the major aspects of the chicken that make it an ideal model system is its development, external of the mother, within the egg. Since the chick develops externally, various variables such as maternal hormonal, metabolic, immunologic, and nutritional changes can be controlled for. This is very difficult in a live-bearing species such as mammals. In addition, the developing embryo can more easily be subjected to various growth conditions to study their effect on development. Two examples are the study of the development of cardiovascular nerves following embryonic hypoxia [27] and the effects on embryonic development due to malnutrition [28]. The physiology of the chicken egg also allows for experiments involving exposure to potentially toxic substances. The air cell of a chicken egg is present to allow the chick to begin breathing without the need to rupture the egg shell [29]. The air cell of chicken eggs has been exploited for the study of the effect of substances such as perfluorooctane sulfonate [30] and cigarette smoke [31] on embryonic development. The *in ovo* chicken has also been used to model embryonic development down to individual signaling molecules, including phosphorylation-mediated signal transduction based developmental signals [32].

Chicken (*Gallus gallus*) was first domesticated around 8000 years ago in Asia [33]. Since then it has been bred for both meat production and egg production, most intensively in the past 40 years. This intensive breeding for production traits makes chickens an excellent species for the study of genetics and proteomics [34]. One of the most significant differences between the birds bred for meat (broilers) and the birds bred for eggs (layers) is the size and growth rate of their muscles, specifically their pectoralis muscle. The rapid development and growth of the chicken makes it an excellent model for the study of muscle development and accretion. Since broilers and layers are evolutionarily similar but display a large difference in muscle growth, they are also ideal for comparative studies of muscle [35].

As zoonotic vectors and reservoirs

Ample evidence has shown that chickens are the source of a number of diseases that affect humans. Two of the most common sources of infection of humans by chicken are viruses such as influenza and bacterial species such as serovars of *Salmonella enterica*.

Avian species are a major reservoir for the influenza virus. While wild avian species such as ducks and geese are often the original source, due to the close contact between humans and chickens it is often from chickens that humans develop the disease. In an outbreak of the H5N1 virus in Hong Kong the virus was found in the feces of 20% of chickens but only 2% of duck and geese feces [20]. In addition, the virus is not only of concern for human health but also the health of the chickens; in the same Hong Kong study there were reports of approximately 75% mortality in influenza infected chicken farms. Research into the mode of infection and the means by which the virus causes disease in chicken may provide valuable insight into influenza. In addition, any potential intervention developed to stop the spread of influenza infection in chicken may have a profound impact of human health. Eliminating the chicken as a reservoir and vector for human influenza would be an important advancement in the fight against a potential future pandemic.

Salmonella infection is a leading cause of disease in humans and can cause symptoms ranging from mild-gastroenteritis to sepsis to typhoid fever [36]. One of the major sources of *Salmonella* infection is food borne bacteria, including chicken. The effects of colonization of the gastrointestinal tract of chickens are quite different than in humans. If a chick more than 5-7 days old is given even a large dose of bacteria it will not develop disease symptoms, despite the bacteria colonizing the chicken gut and the bacteria being shed [37]. Conversely in humans, colonization with *Salmonella* results in inflammation and gastroenteritis, which can lead worsening conditions including severe dehydration, systemic infection and from some serovars, typhoid [36]. This differential response between the two species may shed light on the workings of both the chicken and human immune system, as well as possible treatments for *Salmonella* infection in both species.

Chicken has also been implicated as reservoirs of infection for *Campylobacter jejuni* [38], Newcastle disease virus [39], *Enterococcus faecium* [40] and *Escherichia coli* [41]. From the above list it is clear that host-pathogen studies and immune response research involving the chicken have significant implications not only for animal biology but human health and infectious disease research as well.

Food safety and production

From the previous section it is clear that chicken can be a source of infectious disease in humans. Several of these diseases can be transmitted to humans through the bird's meat. Significant effort is expended by the chicken production industry and scientific researchers attempting to make chicken products safer for the consuming public. These efforts are concerned with every aspect from egg production to butchering techniques.

The increase in the production capacity of chicken, for both eggs and meat, is an area of intense research. It is estimated by the year 2050 the Earth's population will be 9 billion and that food production must increase nearly 70% to keep up with the growing demand [42]. This will mean ever greater demand on breeding the appropriate characteristics into flocks, the reduction in animal disease and increased animal health. In many countries antibiotics are used both to limit disease and promote growth in chickens but the ability to use antibiotics appears to be ending as countries, such as those in the European Union, restrict the use of antibiotics in animal production [43].

The need for intensive scientific research in an attempt to increase food production, the risk of chickens being a reservoir for current zoonoses and emerging pathogens and the suitability of chicken as a model organism, all point to increased use of the chicken in scientific research. This will necessitate new scientific tools and the adaptation of current techniques in the field of chicken research. Currently these tools are limited but a tool to study the chicken kinome is a significant advancement in the ability to study the chicken at an intra-cellular level.

Current tools for chicken kinome research

The tools currently available for the study of the chicken kinome are relatively limited, especially compared to more established research species such as human and mouse. One of the most easy to use methods of determining protein phosphorylation state is the phospho-specific antibody. These are antibodies generated against the region of a protein that undergoes phosphorylation

and is specific for the phosphorylated state of the protein. These antibodies can be used in a number of techniques including western blot [44] and in a microarray format [17]. Unfortunately there are a limited number of antibodies that have been generated specifically for chicken and often one is forced to rely on the cross reaction between a human or mouse antibody and a chicken protein. Since phosphorylation target sites are often quite distinct between species [17] antibody cross reactivity often does not occur.

Activity assays which measure the phosphorylation activity of specific kinases are a tool for kinome study. These assays often have an indirect readout such as ATP depletion or a secondary reaction which is measured over time [45]. The disadvantages of this type of assay is that you are only able to measure the activity of one type of kinase at a time, the advantages are that the assays are often not as species specific as antibody based techniques.

Mass spectrophotometry is a technique that has long been used in proteomics and to study protein post-translational modifications, including phosphorylation. In general the process involves breaking up a protein and determining the sizes of the constituent parts. Computer software analyzes the results and can identify the proteins and any modifications made based on the signal produced. This technique has been widely used to determine phosphorylation sites within a variety of proteins, including chicken proteins [46]. Advantages of this process are it is high-throughput and has the ability to analyze various species. The disadvantages are the specialized equipment and training required to perform the experiment and analyze the data, the difficulty identifying certain proteins and protein isoforms and false positives for phosphorylation are often generated.

Chicken-specific peptide arrays represent a significant advancement in the study of the chicken kinome. Peptide arrays overcome many of the disadvantages of the other techniques in kinome research. A central advantage of the peptide arrays is their species-specific nature and customizability.

Relevant research topics and the kinomic approach

The research potential of chicken-specific kinomics is limited only by the ideas of the researchers interested in applying it. However, there are a number of topics that are currently of scientific interest and are prime candidates for study using this technique.

Infectious disease

The importance of kinases, kinase inhibitors and cell signaling in the context of infectious disease has been well known for decades [47]. Studies using peptide arrays have provided insight into pathogen associated signaling including host responses to CpG oligodeoxynucleotide [48], lipopolysaccharide [7, 49] and even prion [50]. The study of host-pathogen interactions at a cell signaling level can be difficult due to the complexity of the signaling networks as well as attempting to separate general host stress responses from specific immune responses due to infection. The difficulty can be increased in the study of human infectious agents as samples can be difficult or impossible to collect which can necessitate the need to model the human infection. As discussed previously chickens can be a useful model for the study of many biological systems that can then be applied to other species including humans. The mouse is used extensively as a model for human disease but in many cases the mouse responds very differently to an infectious agent than a human [51]. Chickens can be a more appropriate model for human infection in many cases [52]. Conversely in some cases infection of chickens do not cause disease while the same infection of a human host can cause severe disease symptoms [37, 53]. Both the use of chicken as a direct model of human immunity, and the comparative analysis of differential immune response, can be useful in illuminating host biology during an infection.

The development of bacterial resistance is a central concern, not only in the context of human health but for animal health and production as well. There has been an increasing incidence of antibiotic resistant bacterial infections coupled with a reduction in new antibiotic drugs coming onto the market [54]. As a result governments have been increasing the regulatory burden on animal production enterprises and restricting the use of antibiotics for food animals [43]. The control of disease is important in animal production not only for the basic health of the

animal but also for the increased growth rates observed and ultimately a healthier food product. Taking all of this into account alternative methods of controlling disease in animal populations without the reliance on antibiotics is of increasing importance. In the long term it appears increasingly likely that regulations will increase which will severely limit the use of classical antibiotics in chicken production. The increased understanding of the role of kinases in infectious disease may be one avenue of study that could lead to alternative infectious disease control options. A proteomic rather than a genomic approach ensures that any target identified is present during infection. Considering active kinases which are altered during infection insures their presence and role in infection. Many pathogens specifically target kinases and cellular signaling pathways in order to infect and propagate within a host. Examples include, human cytomegalovirus which targets JAK kinases for degradation [55], mumps reduces levels of STAT1 [56], varicella zoster virus reduces levels of Jak2 and STAT1 [57], *Leishmaniadonovani* activates protein tyrosine phosphatase SHP-1 for dephosphorylation and inactivation of Jak2 [58] and *Mycobacterium avium* subsp. *Paratuberculosis* targets the interferon gamma pathway [50]. Kinase inhibitors have been in use as therapeutics including anti-inflammatory drugs which suppress tumor necrosis factor α and interleukin 1β expression function through kinase inhibition [59]. Kinase involvement was determined through the study of the anti-inflammatory activity of pyridinyl-imidazole compounds. These compounds were shown to function through the inhibition of proteins referred to as cytokine-suppressive anti-inflammatory drug-binding proteins. These binding proteins were kinases which activated inflammatory cytokine responses; the kinases are now referred to as isoforms of mitogen-activated protein kinase 14 (Gene Database, ncbi.nlm.nih.gov/gene). Two immunosuppressive drugs, cyclosporine A and rapamycin, function through broad non-specific modulation of the phosphorylation status of the cell: cyclosporine A through inhibition of phosphatases [60] and rapamycin through inhibition of kinases [61]. The targeting of kinases as a means of antibacterial therapy is being increasingly discussed in the literature as typified and reviewed by Cozzone [62].

Gut microbiota

The gut microbiome has received heightened interest of late as a significant potential influence on host health [63]. This recent interest in the gut microbiota as a research topic has led to the understanding that the gut microbiota plays a range of roles in host health besides gastrointestinal infections. The influences of the gut microbiota may be linked to metabolic syndrome, obesity, nutrient absorption, immune response, allergies, cardiovascular health, cancer and even mental health [64]. Through the use of kinomic analysis, both at the gut level and systemically, signaling events being carried from the intestinal lumen to the host can be uncovered. Understanding these signals and potentially influencing them could have significant implications for host health.

Metabolism

The study of metabolism has developed significantly and is considered an 'omic' discipline itself. The metabolome is the complete set of small molecule metabolites found in an organism or sample. The most common methodology used to study the metabolome is mass spectrophotometry. This allows a researcher to see the metabolic processes occurring within a sample by considering the metabolites and pathways that produce them. However, this technique only allows for a static measurement of current metabolite levels within a sample [7]. Metabolism can also be studied via the kinome, measuring active enzymes. The cellular signaling pathways that regulate metabolic processes are controlled by phosphorylation. Knowing the phosphorylation state of the members of the signaling pathways allows one to understand which metabolic processes are being activated, deactivated or changed [7]. It is possible to combine the two techniques, mass spectrophotometry and kinome analysis, to provide a more complete story of host metabolism under given conditions [65].

Biomarker discovery

Biomarkers are an important research tool as a diagnostic, environmental monitor or to determine the efficacy of therapeutic interventions and treatments. Often these biomarkers take the form of a gene or gene product [66] or less often a metabolite [67]. Through the use of kinome

analysis biomarkers can be found at the protein level. If an infection, for example, results in altered cellular signaling, then either the altered signaling molecule or the protein product of the pathway could be used as a biomarker. The phosphorylation of the protein intermediate in a pathway could be monitored through a phospho-assay. The protein product such as a cytokine or hormone could be detected or isolated through various biochemical techniques. The kinomic approach to biomarker discovery has advantages over a genetic approach as one can be more confident in phenotype changes when detecting a protein product or post-translational modification rather than the induction of gene expression. Despite transcription of a gene it cannot always be assumed that the gene will be translated or that the final product will be active and not sequestered or destroyed before it can act. Therapeutic drugs or infectious agents often result in cellular signaling changes throughout the host; this can make it possible to detect kinomic changes in easy to access sources such as blood, even if the target is another tissue [68].

CONCLUSION

Kinome analysis is a powerful method to study a wide variety of physiologically important processes, as phosphorylation-mediated signal transduction is a central part of cell biology. Peptide arrays provide a biologically relevant, high-throughput means of studying the kinome. Species-specific peptide arrays allow the peptide array technique to be applied to a wide variety of species other than human and mouse, including chicken. Chicken is an increasingly important research animal. Central questions remain unanswered in chicken biology and chicken can be used as a model for studying biology which is relevant for other species, in addition chicken is a source of infectious disease which affects humans.

The combination of the chicken, either as a model or the direct study of chicken physiology, with the species-specific peptide array provides an exciting range of research potential. Both will be increasingly important research tools in the coming years.

ABBREVIATIONS

TLR : Toll-like receptor
Treg : T regulatory
ATP : adenosine triphosphate

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