Anthony P. Nicholas
Sanjoy K. Bhattacharya
Paul R. Thompson *Editors*

Protein Deimination in Human Health and Disease

Second Edition

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On behalf of the editors, I wish to dedicate this book to the memory of Kerri Anne Mowen, who sadly passed away at age 41 on February 14, 2016 of a brain aneurysm. Although I had long been familiar with her work, I first met Kerri in 2008 at the FASEB Methylation meeting where we shared our equal passion for both the protein arginine deiminases and protein arginine methyltransferases. What impressed me most about our first meeting was that Kerri was not only whip smart but also a joy to be around. We quickly became collaborators,

and most importantly friends, leading us eventually to cofound Padlock Therapeutics. Kerri's contributions to the PAD field are indelible and include developing both PAD2 and PAD4 knockout mice, helping establish the key role of PAD4 in NETosis, and establishing the importance of PAD4 activity in the initiation versus effector phases of rheumatoid arthritis. Her imprint on the PAD field will long be felt, and her future contributions sadly missed.

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Chapter 1
A History of Deimination Research in Japan: The Founding Fathers

Hidenari Takahara

The research on protein deimination and the enzymes responsible for catalyzing this posttranslational modification started from investigations of the hair follicle and myelin in central nerve system. About 60 years ago, in 1958, the presence of protein-bound citrulline was first reported by Dr. George Rogers (Adelaide University, Australia) in the protein of the inner root sheath (IRS) of hair follicles (Rogers 1958). In order to obtain information about the protein composition of the IRS, he conducted a quantitative amino acid analysis of an acid hydrolysate on sufficient amounts of IRS that were dissected from the vibrissae follicle of rats. At that time, the common method for separating amino acids was paper chromatography, and when applied to the IRS hydrolysates, citrulline was discovered as a distinct ninhydrin-positive spot in an area adjacent to the basic amino acids. About 10 years after Rogers's discovery, Dr. Mario Moscarello (Toronto University, Canada) started an intensive investigation of myelin sheath proteins in the central nerve system. In 1971, he also found the presence of peptide-bound citrulline in myelin basic protein (MBP) using similar methods to Rogers (Finch et al. 1971). Moscarello continued the investigation of MBP until he passed away in 2013, publishing many papers concerning the hyper-deimination of MBP in the pathology of multiple sclerosis. His research career involving the deimination of MBP was described in a eulogy in the first volume of this book series (Nicholas and Bhattacharya 2014).

Although it was unclear as to how the citrulline was incorporated into proteins, the source was thought to be arginine. In 1977, Rogers and colleagues (1977) were the first to conclusively demonstrate that arginine residues were indeed converted to citrulline via a deimination reaction where they combined hair follicle extracts with calcium to promote this reaction. Following this report, research to identify the specific deiminating enzyme was energetically carried out in Japan. In 1979, Dr. Kiyoshi Sugawara (Fig. 1.1; Ibaraki University, Japan) reported the presence of protein-bound

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Fig. 1.1 The founding fathers: *left*, Professor Dr. George Rogers (Adelaide University, Australia). *Center*, Professor Dr. Hidenari Takahara (Ibaraki University, Japan). *Right*, Professor Dr. Kiyoshi Sugawara (Ibaraki University, Japan). Taken at the first International Symposium of Deimination and Skin Biology, April 2009 in Osaka, Japan

citrulline in the epidermal proteins of newborn rat (Sugawara 1979). Successively, he demonstrated the existence of the enzyme that converts arginyl to citrulline residues in the extracts from the newborn rat epidermis (Fujisaki and Sugawara 1981). To assay the enzyme activity, he introduced a colorimetric method using simple synthetic substrates of arginine blocked at the N- and C-terminals as a substrate. In this procedure, high temperatures (over 50 °C) and the presence of dithiothreitol (DTT) greatly enhanced enzyme activity. According to these procedures, he could overcome tedious and laborious work that was needed to measure enzyme activity using an amino acid analyzer. Dr. Sugawara then introduced the logical name peptidylarginine deiminase (PAD) for the enzyme, because it acts on arginine residues embedded in a peptide backbone and is distinct from deiminases that act on free arginine (Fujisaki and Sugawara 1981). In this year, PAD was registered as new enzyme to IUPAC Enzyme Committee and was classified into EC 3.5.3.15.

In 1982, I joined Dr. Sugawara's laboratory. This was just after he obtained a new finding that the extract from rabbit skeletal muscle contains very high deiminase activity, about 120-fold compared to that of the newborn rat epidermis. Since the available amounts of the newborn rat epidermis were very low and the tissue preparations were burdensome, the high abundance of a PAD in rabbit skeletal muscle was an exciting research finding that gave me a tremendous head start to further characterize this enzyme. My first research project at Ibaraki University was to purify the PAD from rabbit skeletal muscle. Very fortunately, I quickly purified the enzyme to homogeneity and determined the chemical, physiochemical, and kinetic properties toward several synthetic arginine derivatives including natural proteins (Takahara et al. 1983). This was the first and most definitive report demonstrating that the

enzyme could catalyze the conversion of arginyl to citulline residues in native protein substrates in vitro. Among the protein substrates examined using this purified PAD, the reaction toward the Kunitz soybean trypsin inhibitor (STI) attracted our attention (Takahara et al. 1985). The effect of the enzyme on STI activity was remarkable as treatment with this PAD rapidly abolished the inhibitory activity of STI without altering its overall conformation; complete inactivation of STI was attained within several minutes at 37 °C. Surprisingly, only the modified arginine residue was the reactive site (or primary contact site) despite the fact that all of the remaining nine arginine residues in STI are exposed on the protein surface (Takahara et al. 1985). This study was first an indication of a biological function for the deimination and biochemical application of PAD. Furthermore, the observation that skeletal muscle PAD showed a high affinity for only the functional arginine residue in STI inspired the idea of an effective affinity adsorbent composed of immobilized STI for PAD purification. Our expectation was fully realized, as a 1800-fold purification with 50% yield was achieved by this affinity column (Takahara et al. 1986). Thereafter, we could supply a sufficient amount of purified rabbit skeletal muscle PAD to other researchers. Although recombinant enzymes from various sources superseded the rabbit skeletal muscle PAD since the latter half of the 1990s, the natural enzyme is still under requisition today. Several earlier experiments conducted with rabbit skeletal muscle PAD elicited important insights into the physiology and pathophysiology of protein deimination. For instance, our collaborative work with Dr. Masaki Inagaki (Aichi Cancer Center, Japan) provided very interesting results. In general, vimentin, an intermediated filament protein, is expressed by various cells and forms a stable, less dynamic molecular network. In 1989, we found that there was a complete loss of filament-forming ability of vimentin after PAD treatment. The enzyme could also deiminate the filaments that had been polymerized and induced filament disassembly. The deimination reduced the isoelectric point of the head domain, in which the positive charge of arginine residues are essential for maintaining the ability to form filaments, resulting in the complete loss of their intermediate filament constructs. Similar results were obtained with other intermediate filaments such as desmin and glial fibrillary acidic protein (GFAP) (Inagaki et al. 1989). Thus, we presumed that deimination of intermediated filaments controls the cytoskeletal network. This hypothesis was verified by several subsequent reports by others in the field. In particular, deiminated vimentin was found in vivo, and this modification triggers structural collapse and promotes apoptosis (Asaga et al. 1998; Hsu et al. 2014). There is also some evidence that deimination of GFAP is a characteristic feature of neurodegenerative diseases (Ishigami et al. 2005).

In parallel with research on rabbit skeletal muscle PAD, we attempted to develop a model system using the mouse/rat for investigation of the physiological function of PAD. From 1988, Dr. Tatsuo Senshu (Tokyo Metropolitan Institute of Gerontology, Japan) started his investigations into the PADs. Together, by 1995, our findings, coupled with those of Senshu's laboratory, established that PAD is widely distributed in many tissues with the notable exception of serum and the location of the enzyme was essentially in cytoplasm. Among the tissues tested thus far, the activity of PAD in the salivary glands, pancreas, and uterus far exceeded those of any other tissues.

Immunohistochemical analyses indicated that the enzyme is preferentially located in acinal cells of the salivary glands and pancreas and in the luminal and glandular epithelia of the uterus (Takahara et al. 1989; Watanabe et al. 1988). Additionally, we noted estrous cycle-dependent changes in enzyme expression in the uterus, with the level being highest and lowest at diestrus (Takahara et al. 1989). Senshu's group also found estrous cycle-dependent change of this enzyme in the rat pituitary gland (Senshu et al. 1989). The expression of PAD in the pituitary and uterus responds adequately to administration with 17β-estradiol (Senshu et al. 1989; Takahara et al. 1992). In the uterus, a remarkable series of events takes place during the estrous cycle. The luminal and glandular epithelia of the uterus at the estrous stage show hyperplasia and vigorously secrete fluid into the lumen. Therefore, PAD may be important for exocrine events, but the physiological roles of PAD in the uterus and pituitary are still unknown.

During investigations of PAD activity in the skin, we had a question: why are the substrate specificities of the skeletal muscle PAD toward several arginine derivatives different from those of the epidermal PAD reported previously (Fujisaki and Sugawara 1981)? Both PADs showed high activity toward the synthetic arginine derivatives blocked at the N- and C-termini (i.e., benzoyl-L-arginine ethyl ester), whereas the skeletal muscle PAD showed very low activity to C-terminal free arginine derivatives such as benzoyl-L-arginine and acetyl-L-arginine. On the other hand, these C-terminal free substrates were comparably processed by epidermal PAD. This question was resolved by our comprehensive work published in 1991 (Terakawa et al. 1991). We compared the elution profiles of the PAD activities of the extracts from several tissues of mouse using anion-exchange chromatography, in which PAD activity was simultaneously measured with the different substrates. As shown in Fig. 1.2, three peaks were eluted upon chromatography of the skin extract. Since each peak showed different substrate specificities, we proposed designating them as peptidylarginine deiminase type I (PAD1), II (PAD2), and III (PAD3) according to the order of elution. The extracts of the skeletal muscle, pancreas, salivary gland, and brain (spinal cord) showed a single peak that corresponded to type II enzyme. Type I enzyme is specifically located in the uterus and epidermal cells, and type III enzyme is present in the hair follicle. These three types of enzyme were not significantly different in catalytic properties, including absolute dependence on calcium ions for activity and the stimulation with DTT. Senshu's group also described the presence of three isozymes in rat tissues and called them "epidermal type, skeletal muscle type, and hair follicle type," which correspond to PAD1, PAD2, and PAD3, respectively (Watanabe et al. 1988). Thereafter, by innovative techniques such as molecular genetics and proteomics, two new PAD isozymes were found in rat epidermis (Yamakoshi et al. 1998)/a keratinocyte cell line (Ishigami et al. 1998) and in the mouse ovary (Wright et al. 2003), and they were named PAD4 and ePAD, respectively. In 1999, Dr. Michiyuki Yamada (Yokohama City University, Japan) and colleagues identified a novel PAD in human myeloid leukemia HL-60 cells, which can induce to differentiate into granulocytes by retinoic acid (Nakashima et al. 1999). By comparison of the amino acid sequence and substrate specificity of HL-60 PAD with those of the four known rat PADs, they

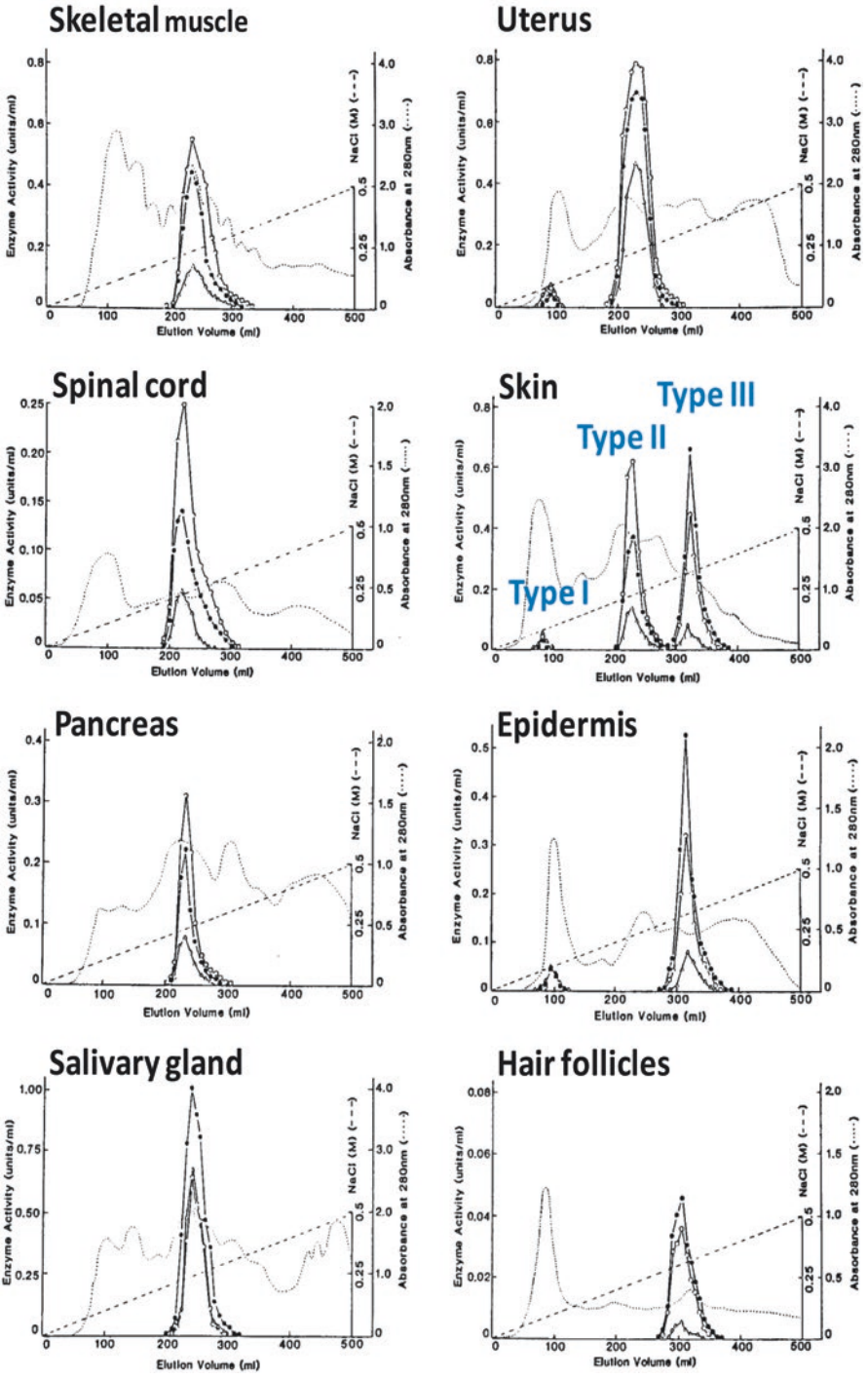

Fig. 1.2 DEAE-Sephacel ion-exchange column chromatography of the PAD activity from various tissues from mouse. The substrates used for measuring PAD activity were Benzoyl-L-Arg-*O*-ethyl ester (*open circle*), Benzoyl-L-Arg (*open triangle*), and protamine (*filled circle*)

concluded that HL-60 PAD did not belong to any PADs and named it PAD5 (Nakashima et al. 1999). However, human PAD5 proved to be the human orthologue of mouse PAD4 (Chavanas et al. 2004), and it was subsequently named PAD4 by the HUGO Gene Nomenclature committee (HGNC). In addition, to avoid confusion, the HGNC recommended that PAD5 remains unused and ePAD be renamed PAD6. In total, it is now recognized that there are five PAD isozymes, i.e., PAD1, PAD2, PAD3, PAD4, and PAD6.

Cloning of the cDNAs for the five PAD isozymes was a historic struggle that stretched from 1989 to 2004. In 1989, Watanabe and Senshu first reported on the cDNA of rat PAD2 and deduced its amino acid sequence (Watanabe and Senshu 1989). Four years later, we revealed at long last in the full nucleotide sequence of mouse the PAD2 cDNA (Tsuchida et al. 1993). Looking back on that time, we had to overcome several obstacles to reach our goal. The N-terminal amino acid sequence of mouse PAD2 was N^{α}-acetyl-Met-Gln-, a sequence which has never previously been reported among N^{α}-acetyl-Met protein. As such, it was difficult to assign the methionine codon (ATG) at the translational start of the cDNA. In 1997, we purified a small amount of PAD3 from newborn rat epidermis by a procedure that included STI-affinity chromatography and carried out peptide mapping by the in situ protease digestion method (Nishijyo et al. 1997). Subsequently, we succeeded in cloning the cDNA and sequenced the full-length cDNA encoding rat PAD3 by RT-PCR and 3′−/5′-RACE methods using synthesized nucleotide primers designed from the internal amino acid sequences (Nishijyo et al. 1997). This was the first report exhibiting the entire amino acid sequence of the isozyme, and by alignment of the PAD2 and PAD3 sequences, we found that a half of C-terminal region was highly conserved, and we predicted that the conserved region was likely responsible for the catalytic activity of this enzyme. This notion was ultimately proved by the excellent works of Dr. Mamoru Sato (Yokohama City University, Japan) and his coworkers in 2004, who determined the X-ray crystal structure of human PAD4 (Arita et al. 2004). These leading researches ultimately resulted in the cloning of the cDNAs for other PAD isozymes. Most cDNA isozymes from the rodent (Yamakoshi et al. 1998; Ishigami et al. 1998; Wright et al. 2003; Watanabe and Senshu 1989; Tsuchida et al. 1993; Nishijyo et al. 1997; Rus'd et al. 1999) and human PAD (Nakashima et al. 1999; Chavanas et al. 2004; Kanno et al. 2000; Ishigami et al. 2002; Guerrin et al. 2003) were established in laboratories in Japan. In addition, we produced the bacterial recombinant PADs of mouse and human in run-up to other laboratories (Ohsugi et al. 1995). The constructed plasmids had a unique DNA linker containing a pair of Shine-Dalgarno sequences and a short preceding cistron inserted into the adjacent 5′-region of the coding region, so that we could obtain a large quantity of the PADs without a sequence tag in bacteria. These recombinant PADs were also easily purified by STI-affinity chromatography and helped a great deal for many investigations (Ohsugi et al. 1995).

In 1992, Senshu and coworkers developed an excellent procedure for the detection of deiminated proteins on membranes or fixed tissues (Senshu et al. 1992). This method involves a three-step process. In the first step, citrulline-containing proteins immobilized on the membrane or fixed tissues are chemically modified. In the

second step, immunoblotting is performed using specific antibodies that were developed against in vitro deiminated histones that were chemically modified in a similar manner. In the third step, citrullinated proteins are visualized using a secondary antibody. This method enabled detection of citrulline-containing proteins at fmol levels, regardless of backbone protein molecules. As known, histones contain a large amount of arginine residues and are highly conserved at the amino acid sequences, regardless of species, which should lead them to the idea for producing the specific antibodies against the chemically modified citrulline. Thereafter, this antibody was called the anti-modified citrulline (AMC) antibody or the "Senshu antibody" and contributed exponentially to the expansion of research on protein deimination and the PADs. Using this immunoblotting analysis, they found that filaggrin and keratin K1/K10 were citrullinated in the epidermis under physiological conditions (Senshu et al. 1995).

For more than 20 years (from 1977 to 1998), most investigations into protein deimination and PAD activity were carried out by only two research groups: ours and Senshu's group in Japan and Moscarrello's group in Canada. Unfortunately, these works were only noticed to a limited extent. There might be two major reasons why protein deimination did not receive a worldwide attention. First, the deiminated proteins were mostly structural proteins in vivo. Second, the PAD reaction is irreversible, and a "peptidyl citrulline-iminase" enzyme has not been discovered. Thus, it was not felt that this modification could reversibly regulate metabolic events and bioprocesses in the same way as phosphorylation/dephosporylation cycles.

However, in 1999, the report of Dr. Guy Serre (University Toulouse III, France) and colleagues developed a new wave that changed the image of the PADs. In this study, Serre and colleagues showed that a "mysterious" antibody present in the sera of patients with rheumatoid arthritis (RA) recognized citrullinated proteins. These antibodies were subsequently named anti-citrullinated protein antibodies (ACPA) (Girbal-Neuhauser et al. 1999). The AMC antibody and purified PAD from rabbit muscle were critical in establishing this finding. Several successive experiments conducted by Serre's laboratory finally identified the antigen recognized in the joints as deiminated α- and β-chains of fibrin (Masson-Bessière et al. 2001). Since the levels of ACPA, including anti-cyclic citrullinated peptide antibodies, are significantly higher in RA patient sera. With high specificity and positive correlation, they are the best diagnostic marker for the disease to date. The direct association between the presence of deiminated proteins and occurrence of RA enticed enormous investigations of protein deimination and PAD. Furthermore, in 2003, the paper of Dr. Akari Suzuki (RIKEN, Japan) and colleagues that described an association of a functional haplotype of the PAD4 gene (*PADI4*) in a Japanese population with RA (Suzuki et al. 2003) further drove an influx of researchers into the field. Once again, in 1999, Yamanda and his coworkers first found human PAD4 in HL-60 cells when cells were induced to differentiate into granulocytes (Nakashima et al. 1999). Their successive finding in 2002, in which PAD4 contains a canonical nuclear localization signal within N-terminal domain and is localized to nucleus, where it deiminated histones H3 and H4, suggested that PAD4 may be a new factor modulating a variety of the nuclear functions dependent on chromatin structure

(Nakashima et al. 2002). These studies also aroused enthusiasm among molecular biochemists who had showed a disinterest in the PADs until then. This is when investigations into protein deimination and PAD activity spread across the world, and the relevant reports increased over the years. To date, PADs are now known to play functional roles in key cellular processes (terminal epidermal and hair follicle differentiations, apoptosis, and gene regulation) under physiological conditions, and dysregulated PAD activity is involved in the pathogenesis of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, Alzheimer's disease, and cancers. So far, these findings were reviewed in many journals including this book series.

For the past decade, the field of deimination research has not so explosively accelerated, but certainly advanced. I believe that these valuable findings are founded on the studies started from their humble beginnings in Japan.

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Chapter 2
Aspects of Peptidylarginine Deiminase Regulation that May Predispose to Autoreactivity Against Citrullinated Proteins

Indira Neeli and Marko Radic

2.1 Summary

Autoimmune diseases represent a long-standing puzzle. In an incompletely understood series of steps, the immune system loses immune tolerance to self and acquires the ability to recognize and respond to defined and characteristic autoantigens. The involvement of an infectious agent has been suspected to trigger this transition, but a specific etiologic stimulus has not been identified. Recent years have seen an evolution in the understanding of events that lead to autoimmunity. A central role has been assigned to posttranslational modifications of autoantigens during the initial, preclinical phase of autoimmune syndromes. In response to various infections or even under sterile inflammatory conditions, the innate immune system activates a characteristic set of enzymatic reactions, including the regulated conversion of certain arginine residues to citrulline residues. The conversion, carried out by the peptidylarginine deiminases (PADs), results in the conversion of arginine residues to citrulline residues in many notable autoantigens. In turn, an important category of autoantibodies, referred to as anti-citrullinated protein antibodies (ACPA), specifically recognizes the citrullinated form of these autoantigens. Thus, the concept is gaining acceptance that diverse infections (or sterile inflammation) result in the citrullination of self-proteins, which—given genetic predisposition or a conducive infectious microenvironment—break tolerance and trigger a self-perpetuating autoimmune process. This chapter highlights aspects of PAD regulation and the development of ACPA in order to propose a unifying principle for the induction of autoimmune disorders.

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2.2 Tolerance and Autoimmunity

Autoimmunity was anticipated as a possible outcome that could arise from the adaptive nature of the immune system (Ehrlich P 1800s). Because the immune system is capable of recognizing almost any foreign molecule, it appeared obvious to Paul Ehrlich that autoreactivity, or "horror autotoxicus," would also predictably appear. Indeed, several dozen human autoimmune disorders are currently recognized as distinct clinical entities, and new disorders continue to be assigned to the category of maladies that arise due to a malfunction of the immune system. Many of these disorders are quite common, such that, for example, rheumatoid arthritis (RA) affects nearly 1 in 100 persons. In aggregate, several percent of the human population develop a serious health condition because of the inappropriate anti-self-reactivity of the immune system.

Conversely, it may be even more astounding that more of us do not suffer from an autoimmune disease, given the enormous rates at which cells of the immune system proliferate and the large numbers of cells that comprise the innate and adaptive immune systems. Moreover, our immune systems are constantly stimulated by microbes that survive in our environment and form part of our extensive microbiota. In view of the continued microbial challenge, it is important to consider ways in which our immune systems distinguish foreign pathogens from self. The mechanism that safeguards against the formation of autoreactive B and T lymphocytes is called immune tolerance. During development and throughout their functional responses, lymphocytes are regulated by active mechanisms that suppress immune responses aimed at molecules that form part of ourselves. The encounter of autoreactive lymphocytes with self-molecules ensures the functional inactivation or correction of the inappropriate B and T cell antigen receptors. Multiple mechanisms are engaged in central tolerance, some of these lead to anergy, deletion, or receptor editing in autoreactive B cells (Radic and Zouali 1996), and others induce cell death of autoreactive T cells in the organs that support lymphopoiesis (Palmer 2003).

The sophistication and power of immune tolerance can best be appreciated by immunization methods in experimental animal species. Standard approaches can readily yield antibodies of high affinity and exquisite specificity for the antigen of choice, provided that the immunogen contains structural motifs (epitopes) that are distinct from the endogenous antigens. For example, a protein that is isolated from the hemolymph of a marine invertebrate, keyhole limpet hemocyanin, is a versatile carrier in numerous immunization experiments. However, if the immunogen of interest is highly related or identical to the animal's own molecular components, multiple immunizations may not elicit an immune response to the intended target. A notable example is provided by the collective experience of frequent but often futile efforts to raise antibodies to various members of the histone protein family (Rubin et al. 1990).

Histones are highly conserved, basic proteins that associate into octamers of two histone H3, two H4, and two each of H2A and H2B monomers (Moudrianakis and Arents 1993). Together these eight histones form the core of a structure that serves to organize approximately 147 base pairs of nuclear DNA into a single nucleosome, the basic unit of chromatin. Histones are among the most ubiquitous, abundant, and

highly conserved proteins in eukaryotes, given that histone primary amino acid sequences vary by less than 1% from humans to yeast. Thus, immunizations of mice or rabbits with histones elicit very little to no immune response due to the strong negative selection of lymphocytes against self-reactivity by immune tolerance. A similar resistance to immunization with DNA is observed, yet both DNA and histones are targets of anti-self-antibodies (Stollar 1971) in an autoimmune disease called systemic lupus erythematosus (SLE). Other conserved and abundant proteins are characteristic autoantigens in other autoimmune diseases. For example, myelin basic protein, a membrane-associated protein of neurons, is recognized by T cells in multiple sclerosis (Martin et al. 1992), insulin is a target of autoantibodies in diabetes (Michels and Nakayama 2010), and collagen or even the immunoglobulin molecule itself is attacked by the immune system in RA (Rowley et al. 2008).

But how does the immune system overcome ("break") immune tolerance and begin an autoimmune attack? Some early illustrative experiments with the multifunctional protein cytochrome c provided important clues. Immune tolerance prevents mice from responding to mouse cytochrome c. However, human cytochrome c, due to only six amino acid variations in sequence from the mouse protein, can induce a B cell response (Lin et al. 1991). Remarkably, following a subsequent immunization with a combination of mouse and human cytochrome c, the mouse B and T cells respond to the mouse cytochrome c protein. The conclusion from those experiments is that small molecular differences between autoantigens and immunogens can break tolerance and induce a self-sustaining autoimmune response, provided that B cells to the altered antigen arise which can present antigen to incompletely deleted T cells.

It was observed that small molecular differences between an immunogen and an autoantigen can also be introduced by various covalent modifications of autoantigens and that such differences can stimulate a response by the immune system. In pioneering experiments, Bill Weigle discovered that chemically modified albumin could break tolerance in rabbits tolerant of the unmodified albumin (Weigle 1962). More recent work from Mark Mamula's laboratory expanded this field of research and showed that isoaspartate racemization of self-antigens, including histones, increases their immunogenicity (Doyle et al. 2013). These experiments established the possibility that posttranslational modification (PTM) of autoantigens may contribute a possible mechanism to disrupt immune tolerance. In turn, studies in a number of laboratories identified autoantibodies that preferentially reacted against the modified form of a variety of autoantigens. This independent evidence solidified the idea that the covalent, enzymatic modifications of autoantigens play a central role in the induction of autoimmunity.

2.3 Discovery of Arginine Deimination

The first identification of citrulline residues in a protein was reported in 1958 by researchers from the Melbourne Wool Research Laboratories examining hair follicles in sheep (Rogers and Simmonds 1958). The discovery was notable for the fact

that citrulline is not incorporated into proteins during translation; thus, it must be introduced into proteins by posttranslational modifications. However, formal proof for this conclusion had to await the discovery of peptidylarginine deiminases (PADs), the enzymes that convert arginine residues in proteins to citrulline residues (Fujisaki and Sugawara 1981). Subsequent research identified additional members of this small protein family, which established the expression of five isoforms of PADs in mammals (Chavanas et al. 2004). PADs are a relatively recent evolutionary adaptation, which are present in vertebrates but absent from other eukaryotic subphyla (Vossenaar et al. 2003).

Research into the structure and function of PADs intensified following the discovery that autoantigens, including myelin basic protein, keratin, and histones, are substrates for PADs (Muller and Radic 2015). These hallmark autoantigens that have high diagnostic value for the detection and prognosis of a variety of difficult to treat autoimmune disorders previously had not been considered to be linked by a common pathway. The discovery of shared PTM between autoantigens in different autoimmune disorders immediately raised expectations that a common underlying mechanism may be at the root of this category of clinical entities. Most notably, a series of elegant studies traced the RA autoreactivity against cells from the oral mucosa to yet another citrullinated antigen. Schellekens and collaborators observed that autoantibodies in RA display a high degree of specificity and sensitivity for peptides derived from filaggrin but only if the arginine residue in these peptides is replaced by citrulline (Schellekens et al. 1998). The diagnostic utility of the serologic test for anti-citrullinated protein antibodies (ACPA) was demonstrated by the observation that ACPA can be detected months or even years ahead of the appearance of clinical manifestations of RA (Johansson et al. 2016). The inclusion of ACPA as classification criteria for RA further boosted basic and applied research into PADs (Liao et al. 2008).

The five PAD isozymes differ from each other in the cell types and subcellular locations where they are expressed and, in turn, by what substrates they modify. PAD1 is expressed in the epidermis and uterus (Terakawa et al. 1991), PAD3 is active in hair follicles (Kanno et al. 2000), and PAD6 is most abundant in the oocyte and during embryonic development (Yurttas et al. 2008). PAD2 and PAD4 are of greatest relevance in cells that comprise the immune system (Vossenaar et al. 2004). Notably, PAD2 is also expressed in the central nervous system and in muscles (van Beers et al. 2013). PAD4 is highly expressed in leukocytes, the white blood cells of the innate immune system, along with other immune cells (Anzilotti et al. 2010). PAD4 is the only PAD with a distinct nuclear localization sequence, and thus, it is the probable isozyme in charge of modifying nuclear proteins, including histones (Nakashima et al. 2002). Accordingly, activation of PAD4 makes important contributions to chromatin structure modifications that regulate gene expression (Cuthbert et al. 2004; Wang et al. 2004). However, PAD2 has also been implicated in the modification of histones (Zhang et al. 2012), whereas PAD4 may also be active in the cytoplasm and even on extracellular substrates (see below).

All PADs require calcium for enzymatic activity, and maximal activity may require extracellular calcium influx to supplement intracellular calcium stores. Calcium ions organize the overall PAD structure and shape the conformation of the active site, as determined by X-ray diffraction patterns of PAD4 obtained in the presence and absence of the divalent cations (Arita et al. 2004). PADs are relatively promiscuous enzymes because they accept substrates with different amino acid sequences, provided the target arginine is preceded by a residue with a small side chain at the R-2 position and the polypeptide chain flanking the arginine can fold into a tight beta turn (Arita et al. 2006). Catalysis, which consists of the hydrolysis of the guanidino group on arginine to yield the ureido group on citrulline (Fig. 2.1), leads to the release of ammonia, the loss of arginine's positive charge, and the nearly exact gain of 1 Da in mass by the citrulline-containing product (Rohrbach et al. 2012b). Although PAD4 can accept methylated arginine as a substrate, it prefers unmodified arginine by a factor of over 100:1 in an in vitro reaction (Kearney et al. 2005; Raijmakers et al. 2007). Therefore, biochemically, one consequence of arginine citrullination is to preclude any further arginine modifications (Thompson and Fast 2006).

As the evidence for autoantibodies to citrullinated antigens accumulated, it became clear that useful applications would emerge from the development of PAD inhibitors. Insights into the conformation of the PAD4 active site were starting points for the design of different versions of PAD4 inhibitors. Work spearheaded by the Thompson lab used various chemical approaches to screen and confirm the efficacy, specificity, bioavailability, and mode of action of various compounds (Bicker and Thompson 2013). One of the earliest inactivators that were effective at inhibiting the action of several PAD enzymes was *N*-alpha-benzoyl-*N*5-(2-fluoro-1-iminoethyl)-L-ornithine amide, or F-amidine, which became available about 10 years ago (Luo et al. 2006), and was soon followed by several other irreversible and selective inhibitors (Knuckley et al. 2010). For example, a natural compound with antibiotic properties, streptonigrin, was identified as a selective PAD4 inhibitor (Dreyton et al. 2014), whereas the inorganic dye used in histology, ruthenium red,

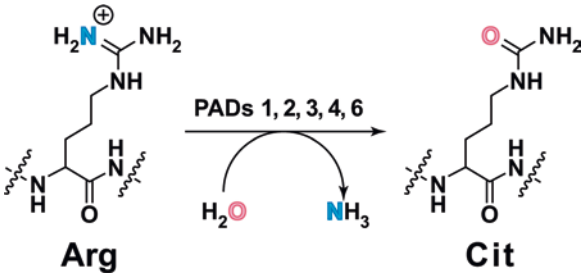

Fig. 2.1 Diagram of peptidylarginine deiminase (PAD)-mediated conversion of arginine to citrulline. One of five PADs converts arginine to citrulline by using oxygen from water and releasing nitrogen as ammonia

was a selective inhibitor of PAD2 (Lewallen et al. 2014). Ruthenium red binds to PAD2 at a site that normally accepts calcium, and thus, it prevents the activation of the enzyme. These inhibitors and inactivators are of immense value for determination of PAD activity and its consequences in vivo and in vitro.

Mounting evidence places PAD4 at the center of attention in events shaping the interactions between the innate and adaptive immunity. In particular, research indicates that PAD4 activation is intimately involved in the initial stimulation and subsequent tissue damage associated with autoimmune diseases. Nevertheless, additional open questions remain and deserve further attention. For example, although PAD4 can auto-citrullinate (Andrade et al. 2010; Mechin et al. 2010), the precise consequences of this modification are unclear, as the modified PAD shows little change in enzymatic activity on selected in vitro substrates (Slack et al. 2011). Clearly, one possibility in vivo is that auto-deimination may affect the binding with other interacting partners of the enzyme. More broadly, several unsolved questions of PAD4 regulation remain, and efforts at finding answers will drive much ongoing and future research. Some of the questions are highlighted in Box 2.1.

Box 2.1: Unsolved Questions in Cell Biology of PADs

1. Is PAD4 enzymatic activity controlled by other interacting proteins, many of which are yet to be identified?
2. Do PAD4 modifications regulate the access of calcium to its five binding sites?
3. What principles determine PAD4 recognition of other proteins and the cellular location of the enzyme?

2.4 Role of PAD4 in Innate Immune Responses

PAD4 supports fundamental functions in neutrophils, the most abundant type of granulocytes in blood. The PAD4 gene is expressed relatively late during neutrophil maturation but reaches high expression levels during a short window preceding the release of neutrophils from the bone marrow (Theilgaard-Monch et al. 2005). Even though PAD4 clearly can be transported into the nucleus and its most abundant cellular substrates are core histones, large quantities of PAD4 are also packaged into ficolin-1-rich neutrophil granules (Rorvig et al. 2013). The issue of subcellular localization can be addressed with the use of specific antibodies raised against PAD4. Figure 2.2 shows the results of probing human peripheral blood neutrophils with a monoclonal antibody to PAD4 (kind gift of Prof. Katsuhiko Nakashima). Using a preparation of unstimulated neutrophils, it can be seen that cells exhibit heterogeneous staining patterns, in which some cells have abundant nuclear PAD4, whereas others have a more cytoplasmic PAD4 distribution. In addition, particular cells exhibit a patchy nuclear distribution, perhaps indicating that PAD4 shows preference for certain chromosomal domains. The resolution of the confocal microscope does not allow the precise localization of PAD4 to cytoplasmic granules, but

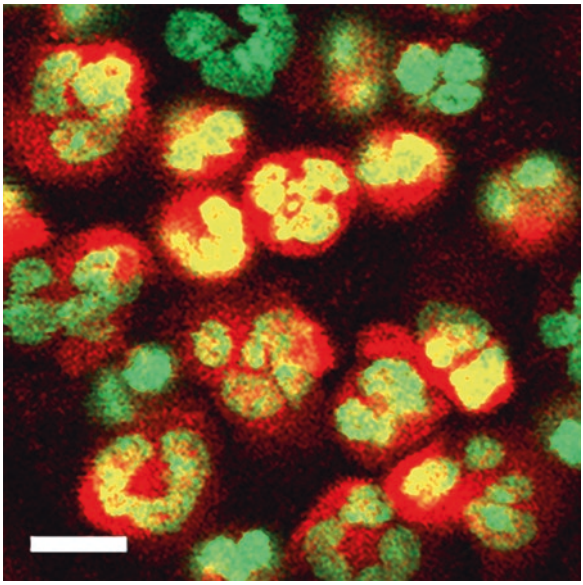

Fig. 2.2 Detection of PAD4 in purified human blood neutrophils. Human neutrophils were isolated from blood and purified using standard conditions. Fixed cells were permeabilized and incubated with a monoclonal antibody to PAD4 (kind gift from Dr. Nakashima, Japan). Antibody binding was visualized by secondary anti-mouse antibodies shown in *red*, and nuclear DNA was detected with *Sytox Green*. The overlap between the colors yields *yellow*. In this preparation, neutrophils were heterogeneous, and PAD4 was localized to both nuclei and cytoplasm. Bar is equivalent to 10 μm

that assignment is consistent with biochemical fractionation experiments carried out by Niels Borregaard's laboratory in Copenhagen.

The protective function of neutrophils in the immune system is to act as sentinels for infections or inflammation. As white blood cells, neutrophils circulate throughout the body, yet they are exquisitely sensitive to any local signs of inflammation. The presence of IL-8 (CXCL8) and expression of selectins on endothelial cells lining the blood vessels induce neutrophil attachment to the vessel wall (Riese et al. 2014) and their extravasation (migration) into tissues. Neutrophils express metalloproteases such as MMP-9 which transiently dissociate tight junctions that bind endothelial cells to each other, thus generating gaps that neutrophils exploit to squeeze through the endothelium and reach the inflamed tissues (McColl et al. 2008). Once in tissues, neutrophils convert into highly motile cells capable of chemotaxis toward increased concentrations of microbial patterns such as f-MLP and various cytokines, such as IL-1 or TNF. Neutrophils also migrate toward signals from damaged cells, such as ATP, ADP, and related molecular messengers that promote inflammation (McDonald et al. 2010). Neutrophils integrate these diverse signals by expression of multiple cell surface receptors that operate in a hierarchical system of dominant vs. supplementary signaling cascades (Heit et al. 2002; Mocsai et al. 2015).

The functional transition of a neutrophil from passive transport in the blood to an active, directional movement in tissues is mirrored in a notable increase in gene transcription. In part, the upregulation of gene expression reflects the activation of cell surface adhesion molecules, such as Mac1 integrins, which are important in the process of extravasation as well as in the subsequent migration in tissues (Kobayashi et al. 2002). The coordinated migration of neutrophils in tissues has been described as "swarming" which can be viewed in vivo following a focused tissue injury (Lammermann et al. 2013). Expression of Mac1 is required for the formation of tight clusters that comprise the center of swarms and assist in the repair of damaged extracellular matrix components. A variety of gene products is induced, most notably several pro-inflammatory cytokines and chemokines that serve to condition the tissue microenvironment and promote the migration of other cell types to the site of inflammation (Nathan 2006).

PAD4 expression is induced by several of the signals that promote inflammation, including TNF (Neeli et al. 2008) and IL-8 (Gupta et al. 2005). Moreover, PAD4 expression is stimulated by neutrophil adhesion to extracellular matrix components via Mac1 and is sustained by an active and functional cytoskeleton (Neeli et al. 2009). Most importantly, PAD4 expression is strongly induced by substances emanating from pathogens or by direct contact with microbes. Bacteria, fungi, and viruses that upregulate PAD4 activity include clinically important pathogens such as *Staphylococcus aureus* (Kolaczkowska et al. 2015) and *Shigella flexneri* (Li et al. 2010). It is therefore tempting to say that PAD4 activation begins with the stimulation of neutrophils in the blood, progresses through the stages of active migration toward a site of infection, and culminates during the precise moment that contact with an invasive pathogen is made. Upon encounter with microbes, the neutrophils may induce alternative responses. Neutrophils are active phagocytes capable of the internalization and destruction of a pathogen. Alternatively, neutrophils can release bactericidal products and reactive oxygen species (ROS). Although the precise role of PAD4 during phagocytosis is not known, it has been reported that various secreted cytokines and chemokines are modified by deimination (Proost et al. 2008; Moelants et al. 2013). It thus appears that PAD4 is intimately involved in essential functions of the innate immune system, for which it regulates and fine-tunes many complementary but independent processes.

2.5 PAD4 and NETs

An additional and unexpected innate defense mechanism was initially described in 2004 (Brinkmann et al. 2004). These authors observed that in response to various bacterial pathogens, neutrophil cell membranes rupture and release nuclear chromatin into the cellular surroundings. The released chromatin forms a matrix that was named a "neutrophil extracellular trap" (NET) because it has the ability to bind and immobilize the pathogens that it contacts. Subsequent studies have more carefully examined the steps in NET release (Fuchs et al. 2007). Morphologically, it was observed that the nuclear chromatin relaxes, and, as a result, the lobed nucleus

expands until it is roughly spherical. Thereupon, the nuclear envelope distends until gaps appear, the cytoplasmic granules break open, and granule contents associate with the chromatin that breaks out from the confines of the nucleus. Ultimately, the plasma membrane ruptures and NETs that consist of nuclear chromatin and associate with several granule constituents, such as myeloperoxidase and elastase (Papayannopoulos et al. 2010), are deployed. It is assumed that the granule contents such as proteases and antibacterial peptides, which decorate the NETs, contribute to their bactericidal potential. Interestingly, certain strains of bacteria express nucleases that allow them to escape from neutrophil traps (Buchanan et al. 2006). In vivo, these bacteria are more virulent than variants that are deficient in nuclease production.

Separate experiments have provided evidence for the release of NETs in response to certain viruses and fungal infections. Infections with poxviruses identified a protective function of NETs in the microvessels of the liver (Jenne et al. 2013). In that study, NETs were visualized in vivo and provided effective containment of viruses in liver sinusoids which protected the body from viral dissemination. Interestingly, neutrophils also release NETs in response to fungi such as *Aspergillus* (Gazendam et al. 2016) and *Candida* (Byrd et al. 2013). In fact, the relative size of a pathogen may determine, in part, whether the neutrophil will release NETs or undergo an alternative innate immune defense mechanism (Branzk et al. 2014). Cell wall glucans of *Candida* are also effective inducers of neutrophil swarming in vitro (Byrd et al. 2013). These and other in vivo studies determined that there is a type of NET release that does not result in the massive rupture of the cell but, in fact, leaves the neutrophil able to continue chemotaxis (Yipp et al. 2012). It was further determined that this alternative form of NETs may be composed of mitochondrial DNA (Yousefi et al. 2009). If so, the mitochondrial NETs may lack histones and perhaps other granule-derived bactericidal components of classical NETs. Clearly, important characteristics of the mitochondrial nucleoid NETs and their ability to damage or destroy pathogens remain to be established.

In the classical form of NET release, numerous independent and consistent studies demonstrated that PAD4 carries out an essential function. In pioneering studies, our lab showed that various inflammatory stimuli induce histone deimination and identified deiminated histones as integral components of NETs (Neeli et al. 2008). Subsequent studies confirmed these results (Wang et al. 2009) and established that PAD4 activity is essential for the regulated release of NETs, as neutrophils deficient in PAD4 fail to deploy extracellular chromatin (Li et al. 2010; Rohrbach et al. 2012a). These results are consistent with evidence that PAD4 inhibitors are effective at blocking NET release (Lewis et al. 2015). However, it is not clear how PAD4 contributes to NETosis. It is plausible, albeit unproven, that PAD4 contributes an essential function for NET deployment by modifying arginine residues in histones. The amino termini of core histones extend from the nucleosome core particle in unstructured fashion. Each of the four core histones has extended amino termini of about 18–25 residues in length that contain numerous positively charged arginine and lysine side chains (Radic and Muller 2013). Through these extensions, each of the eight histones in a nucleosome can contribute to bind adjacent nucleosomes into

condensed and relatively inert chromatin. A fifth histone, the linker histone H1, does not form part of the nucleosome core particle yet contributes in an important way to chromatin structure. Histone H1 binds DNA at the entry and exit points from the nucleosome and thus controls the angle of the linker DNA that connects adjacent nucleosomes. In that way, H1 is at a crucial position to regulate chromatin structure (Izzo and Schneider 2016). The histone tails, together with H1, regulate structural transitions in chromatin, which facilitate access of transcription factors, RNA polymerases, and other proteins to particular DNA sequences. Many histone PTMs serve to organize chromatin according to the functional needs of the cell.

By converting arginine residues to citrulline residues, PAD4 removes the positive charge from the amino termini of core histones (Fig. 2.1) and thus diminishes the attractive forces between histones and DNA. As a result, histone deimination loosens the structure of chromatin. Yanming Wang and colleagues used defined chromatin templates to show their structural relaxation upon treatment with PAD4 (Wang et al. 2009), and a similar transition may provide the force that expands the nucleus and ultimately ruptures the nuclear envelope to release NETs. In their sequence, H1 histones contain a large number of lysine residues but only a few arginine residues. Therefore, we asked whether one or more of these arginines are deiminated by PAD (Dwivedi et al. 2014). We prepared highly modified histone H1 and subjected it to tandem mass spectrometry. Peptide analysis determined that the most highly conserved arginine, R53, in the winged helix domain of H1, is a substrate of PAD4. In independent studies by the laboratory of Tony Kouzarides in Cambridge, deimination of R53 was reported to occur during transcriptional reprogramming that accompanies the development of cell lineages from pluripotent stem cells (Christophorou et al. 2014). The deimination of H1 by PAD4 thus was shown to play a crucial and conserved role in the developmental program of higher eukaryotes. Strikingly, the same H1 modification that facilitates global changes in gene expression also was co-opted toward a unique mechanism of innate immune response.

The myriad of stimuli that lead to NET release and the potential existence of different forms of NETs make it difficult to identify the signaling pathways that participate in the activation of PAD4. Signals from Gram-positive bacteria, including lipopolysaccharide (LPS) acting on the Toll-like receptor 4 (TLR4), may transmit signals via MyD88 and its associated catalytic subunits to IRAK1 (Huang et al. 2015). Through the activation of distinct IKK subunits, the pro-inflammatory axis of NFkappaB is engaged, leading via MEK1 to the further activation of ERK1 and ERK2 (Yu et al. 2015). Alternatively, it was reported that FcgammaRIIIb, acting through TAK1, leads to the activation of ERK1/ERK2 (Aleman et al. 2016). Additional feed-forward signals may involve activation of PLCgamma and the formation of its messenger IP3, followed by calcium release from endogenous ER stores (Numaga et al. 2010). Alternatively, a calcium-activated potassium channel may directly engage signals leading to NETosis (Douda et al. 2015). Calcium could act as an additional signal by activating PKC subunits, which have been shown to have a direct effect on NET release. Our own studies revealed an unexpected complexity of PKC involvement in NETosis. Experiments with an inhibitor

of classical PKC, chelerythrine, as well as a structurally related compound, sanguinarine, demonstrated that classical PKC enzymes may block activation of PAD4, yet an atypical PKC, most likely PKCzeta, exerts an activating role upstream of PAD4 (Neeli and Radic 2013). The opposing effects of two PKC isoforms argue for very precise regulation of PAD4 in neutrophils. Through as yet incompletely understood mechanisms, these enzymes contribute to the disruption of granule and nuclear membranes, chromatin relaxation, and, ultimately, NET release. Some of the remaining questions regarding the regulation of NET release are summarized in Box 2.2.

The activation of PAD4 can most easily be seen by monitoring histone deimination (citrullination), for which specific antibody reagents are commercially available. However, discovery of new substrates will require the availability of additional approaches. One method to detect the activation of PAD4 in cells is by isolating neutrophil proteins and exposing them to a compound that selectively reacts with the ureido group of citrulline. Under acidic conditions, phenylglyoxal reacts with citrullines to form a covalent bond that is stable during subsequent manipulations at neutral pH (Lewallen et al. 2015). By conjugating rhodamine to the phenylglyoxal probe (Rh-PG), citrulline-containing proteins become fluorescently labeled. We have used such an approach to explore the diversity of neutrophil proteins that are substrates for PAD4 (Fig. 2.3).

The results demonstrate that, in purified human neutrophils, certain proteins react with Rh-PG following incubation in calcium-containing buffer (lanes 1). However, the intensity of Rh-PG reactivity increases following neutrophil activation with ionophore (lanes 2). In particular, histones in ionophore-activated neutrophils become quite reactive, indicating deimination by PAD4. An increased intensity of reactivity is observed following ionophore stimulation in the presence of a classical PKC inhibitor (lanes 3). Purified calf thymus histones that were deiminated in vitro by recombinant PAD4 (kind gift from Paul Thompson) indicate the migration of histones (lane labeled H). The specificity of Rh-PG labeling is tested by comparison to the reactivity of antibodies reactive against modified citrulline (lane MC). There is extensive agreement between these two approaches (compare lanes 3 to MC). An alternative method could use a mouse monoclonal antibody, F95, which was raised against a deca-citrulline peptide and which reacts with citrulline-containing antigens (Nicholas and Whitaker 2002). Each of these should be added to the list of reagents that will play a crucial role in the further identification of PAD4 functions in NETosis.

Box 2.2: Unsolved Issues of PAD4 Activation in Classical NETosis

1. By using a defined NETosis stimulus, the precise series of contingent activation steps leading to PAD4 activity need to be established.
2. The precise relation between PAD4 activation and nuclear chromatin relaxation should be demonstrated.
3. The possible activation of PAD4 during the mitochondrial NET release and/or swarming should be examined.