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Phosphoinositide 3-Kinase in Nitric Oxide Synthesis in Macrophage

*CRITICAL DIMERIZATION OF INDUCIBLE NITRIC-OXIDE SYNTHASE**

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Phosphoinositide 3-kinase (PI3K) has important functions in various biological systems, including immune response. Although the role of PI3K in signaling by antigen-specific receptors of the adaptive immune system has been extensively studied, less is known about the function of PI3K in innate immunity. In the present study, we demonstrate that macrophages deficient for PI3K (p85α regulatory subunit) are impaired in nitric oxide (NO) production upon lipopolysaccharide and interferon-γ stimulation and thus vulnerable for intracellular bacterial infection such as *Chlamydophila pneumoniae*. Although expression of inducible nitric-oxide synthase (iNOS) is induced normally in PI3K-deficient macrophages, dimer formation of iNOS protein is significantly impaired. The amount of intracellular tetrahydrobiopterin, a critical stabilizing cofactor for iNOS dimerization, is decreased in the absence of PI3K. In addition, induction of GTP cyclohydrolase 1, a rate-limiting enzyme for biosynthesis of tetrahydrobiopterin, is greatly reduced. Our current results demonstrate a critical role of class IA type PI3K in the bactericidal activity of macrophages by regulating their NO production through GTP cyclohydrolase 1 induction.

Phosphoinositide 3-kinase (PI3K)[2] is a key enzyme in various signal transduction pathways, such as cytoskeletal rearrangement, survival, and cell cycle progression (1). PI3Ks are classified into three groups according to their structure and substrate specificity. Of those groups, class IA heterodimeric PI3Ks, with each enzyme consisting of a catalytic subunit and a regulatory subunit, are involved in receptor-mediated signaling in the immune system. We and others (2, 3) have developed mice lacking the gene for p85α, the most abundant and ubiquitously expressed regulatory subunit of the class IA PI3Ks, and reported the significance of these enzymes in B cell differentiation and activation (4–6). We found that class IA PI3Ks are essential for development and activation of B lymphocytes (5, 6), granulocytes (7), platelets (8), and mast cells (9). Little is known, however, about the function of PI3K in the innate immune response (10–12).

Innate immunity is critical in self-defense for the host against microbial infection, in which macrophages play central roles in the initiation of inflammation by producing proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6, and bactericidal radical effector molecules such as nitric oxide (NO) and reactive oxygen species. The considerable susceptibility to intracellular bacteria and protozoa in mice deficient in inducible nitric-oxide synthase (iNOS) (13–15), indicated that production of NO is important in host self-defense against intracellular microbes (16). NO is generated from arginine by three isotypes of NOS family enzymes, which are controlled by three independent genes: NOS1, NOS2, and NOS3 (17). Among them, iNOS (NOS2) is responsible for NO production in macrophages. Ligands for Toll-like receptors as well as cytokines can stimulate macrophages to induce iNOS, whose expression depends on transcription factors such as nuclear factor-κB (NF-κB), AP-1, STAT1, IRF1, and NF-IL6 (18–22). NO generation by iNOS is subject to multiple levels of regulation, including transcriptional, translational, and post-translational regulation. Homodimerization is essential for iNOS activity, and dimerization requires cofactors such as calmodulin, FAD, FMN, NADPH, and heme (23). The dimers are further stabilized by binding of tetrahydrobiopterin (BH4) and the substrate arginine (23). In contrast, NAP110 and kalirin associate with iNOS and thereby inhibit iNOS dimerization (24, 25). Cytosolic arginine content is affected by many factors including arginase and cationic amino acid transporter 2 (CAT2) membrane amino acid transporter proteins (26–29). Furthermore, iNOS protein itself is actively degraded in a proteasome-dependent pathway (30). Production of NO is thus regulated by many factors (31).

PI3K inhibitors have been useful for the investigation of the effect of PI3K on NO production in macrophage cell lines (32–34). However, results from different reports have not been consistent. To clarify the precise function of PI3K in the regulation of NO production and mechanisms of host defense against microbes, we utilized our p85α-deficient mice as PI3K knock-out mice. In the present study, we found that PI3K-deficient macrophages produced less NO than wild-type macrophages after stimulation of with interferon-γ (IFN-γ) and lipopolysaccharide (LPS), and that these macrophages were further susceptible to bacterial infection.

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[2] The abbreviations used are: PI3K, phosphoinositide 3-kinase; CAT2, cationic amino acid transporter 2; LPS, lipopolysaccharide; GCH1, GTP cyclohydrolase; BH4, tetrahydrobiopterin; NOS, nitric-oxide synthase; iNOS, inducible NOS; IFN-γ, interferon-γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; RT-PCR, reverse transcriptase PCR; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

Developmental Stage-Dependent Collaboration between the TNF Receptor-Associated Factor 6 and Lymphotoxin Pathways for B Cell Follicle Organization in Secondary Lymphoid Organs[1]

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Signal transduction pathways regulating NF-κB activation essential for microenvironment formation in secondary lymphoid organs remain to be determined. We investigated the effect of a deficiency of TNFR-associated factor 6 (TRAF6), which activates the classical NF-κB pathway, in splenic microenvironment formation. Two-week-old TRAF6-deficient mice showed severe defects in B cell follicle and marginal zone formation, similar to mutant mice defective in lymphotoxin (Lt) β receptor (Ltβ R) signal induction of nonclassical NF-κB activation. However, analysis revealed a TRAF6 role in architecture formation distinct from its role in the early neonatal Lt signaling pathway. Ltβ R signal was essential for primary B cell cluster formation with initial differentiation of follicular dendritic cells (FDCs) in neonatal mice. In contrast, TRAF6 was dispensable for progression to this stage but was required for converting B cell clusters to B cell follicles and maintaining FDCs through to later stages. Fetal liver transfer experiments suggested that TRAF6 in radiation-resistant cells is responsible for follicle formation. Despite FDC-specific surface marker expression, FDCs in neonatal TRAF6-deficient mice had lost the capability to express CXCL13. These data suggest that developmentally regulated activation of TRAF6 in FDCs is required for inducing CXCL13 expression to maintain B cell follicles. ***The Journal of Immunology*****, 2007, 179: 6799–6807.**

Secondary lymphoid organs provide an environment that facilitates interactions among lymphocytes and between lymphocytes and APCs, interactions that are essential for initiation of an effective immune response. Although the structures and the development of the secondary lymphoid organs during ontogeny have been studied in detail, the molecular mechanisms involved in their development and organization remain unclear (1–4). Studies with gene-targeting techniques revealed that lymphotoxin (Lt)[3] β receptor-mediated signaling is required for the development of the secondary lymphoid organs including lymph nodes and Peyer's patches (2, 5, 6). Intracellular signaling from Lt β receptor (Ltβ R) induces transcriptional activation by members of the NF-κB family through both the canonical and noncanonical pathways (7, 8). Deficiency in NF-κB-inducing kinase (NIK) or RelB, which are essential for the noncanonical pathway, results in the absence of lymph nodes and Peyer's patches in mice (7, 8). These results suggest that the noncanonical NF-κB activation pathway plays a critical role in the development of these secondary lymphoid organs (8, 9).

The microarchitecture of the splenic white pulp, in which the immune response against blood-borne Ags initiates, requires Ltβ R signaling for development (6, 10). The white pulp in naive adult mice is divided into the B cell follicles, the T cell zone, and the marginal zone. The regulated interaction between lymphocytes and stromal cells is essential for development of these organized structures in the white pulp. For development of B cell follicles, the interaction between B cells and FDCs, a subset of stromal cells in lymphoid follicles, is crucial (11). Stimulation of Ltβ R on FDCs by B cell surface-anchored Ltα1β2 complex induces FDC expression of CXCL13, a chemokine that attracts B cells. Interaction of CXCL13 with its receptor, CXCR5, on B cells increases cell surface expression of Ltα1β2, which in turn stimulates the Ltβ R on FDCs to express CXCL13. This process provides a positive feedback loop that is essential for the development of primary B cell follicles (12).

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[3] Abbreviations used in this paper: Lt, lymphotoxin; Ltβ R, Lt β receptor; NIK, NF-κB-inducing kinase; TRAF6, TNFR-associated factor 6; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TRANCE, TNF-related activation-induced cytokine; FDC, follicular dendritic cell; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PN, postnatal; MEF, mouse embryonic fibroblast; MOMA, anti-monocytes/macrophases; JNK, Jun-N-terminal nucleotide kinase; ER-TR9, anti-mouse SIGN-R1 (clone ER-TR9).

Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells

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Summary

The membrane-anchored matrix metalloproteinase MT1-MMP (also known as Mmp14) plays a key role in the angiogenic process, but the mechanisms underlying its spatiotemporal regulation in the in vivo setting have not been defined. Using whole-mount immunohistochemical analysis and the *lacZ* gene inserted into the *Mmp14* gene, we demonstrate that MT1-MMP vascular expression in vivo is confined largely to the sprouting tip of neocapillary structures where endothelial cell proliferation and collagen degradation are coordinately localized. During angiogenesis in vitro, wherein endothelial cells are stimulated to undergo neovessel formation in the presence or absence of accessory mural cells, site-specific MT1-MMP expression is shown to be controlled by crosstalk between endothelial cells and vascular smooth muscle cells (VSMC). When vessel maturation induced by VSMCs is inhibited by introducing a soluble form of the receptor tyrosine kinase Tek, MT1-MMP distribution is no longer restricted to the endothelial tip cells, but instead distributes throughout the neovessel network in vitro as well as ex vivo. Taken together, these data demonstrate that vascular maturation coordinated by endothelial cell/mural cell interactions redirects MT1-MMP expression to the neovessel tip where the protease regulates matrix remodeling at the leading edge of the developing vasculature.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/9/1607/DC1

Key words: MT1-MMP, Angiogenesis, Endothelial cells, Mural cells, Type I collagen

Introduction

Angiogenesis, the growth of new vasculature from pre-existing capillaries, is an important component of normal growth, tissue repair and neoplasia (Carmeliet, 2005; Davis and Senger, 2005). In both normal and tumor stroma, type I collagen is the most abundant component of the extracellular matrix and serves not only as a key scaffolding material but also as a physical barrier for new vessel formation (Chun et al., 2004; Seandel et al., 2001; Davis and Senger, 2005; Saunders et al., 2006). As multiple matrix metalloproteinases (MMPs), including MMP-1, MMP-2, MMP-8, MMP-13 and the membrane type 1 MMP MT1-MMP (also known as MMP-14) display type I collagenolytic activities in vitro (Brinckerhoff et al., 1987; Ohuchi et al., 1997; Hotary et al., 2000; Atkinson et al., 2001), several of these enzymes have been proposed to play important roles in regulating angiogenesis in vivo (Zhou et al., 2000; Chun et al., 2004; Ling et al., 2004; Zijlstra et al., 2004). Recently, however, increasing evidence has begun to accumulate that MT1-MMP serves as the dominant pericellular collagenase during angiogenesis (Zhou et al., 2000; Koike et al., 2002; Chun et al., 2004; Saunders et al., 2006). Whereas neovessel formation is severely impaired in tissues rich in type I collagen in *Mmp14*-null mice (Zhou et al., 2000), little is known with regard to the factors that regulate vascular MT1-MMP expression in the in vivo setting. To this end, we have established a transgenic mouse line wherein the *lacZ* gene (encoding β-galactosidase) was inserted into the endogenous *Mmp14* gene to monitor its transcriptional regulation (MT1-MMP$^{+/lacZ}$ mice). In combination with an ex vivo angiogenesis model using murine tissues cultured under 3-dimensional (3D) conditions, we now demonstrate that MT1-MMP expression is largely confined to the sprouting tip of the developing vasculature, a site where cell proliferation and focal degradation of collagen proceed preferentially. Further, we find that MT1-MMP protein expression is downregulated throughout the vessel stalk by surrounding mural cell

Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction

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One of the central tasks of stem cell biology is to understand the molecular mechanisms that control self-renewal in stem cells. Several cytokines are implicated as crucial regulators of hematopoietic stem cells (HSCs), but little is known about intracellular signaling for HSC self-renewal. To address this issue, we attempted to clarify how self-renewal potential is enhanced in HSCs without the adaptor molecule Lnk, as in Lnk-deficient mice HSCs are expanded in number >10-fold because of their increased self-renewal potential. We show that Lnk negatively regulates self-renewal of HSCs by modifying thrombopoietin (TPO)-mediated signal transduction. Single-cell cultures showed that Lnk-deficient HSCs are hypersensitive to TPO. Competitive repopulation revealed that long-term repopulating activity increases in Lnk-deficient HSCs, but not in WT HSCs, when these cells are cultured in the presence of TPO with or without stem cell factor. Single-cell transplantation of each of the paired daughter cells indicated that a combination of stem cell factor and TPO efficiently induces symmetrical self-renewal division in Lnk-deficient HSCs but not in WT HSCs. Newly developed single-cell immunostaining demonstrated significant enhancement of both p38 MAPK inactivation and STAT5 and Akt activation in Lnk-deficient HSCs after stimulation with TPO. Our results suggest that a balance in positive and negative signals downstream from the TPO signal plays a role in the regulation of the probability of self-renewal in HSCs. In general, likewise, the fate of stem cells may be determined by combinational changes in multiple signal transduction pathways.

c-mpl | p38 MAPK | STAT5 | Akt

Manipulation of stem cell self-renewal is a necessity for the development of stem cell-based regenerative and transplantation medicine. To this end, we need to understand molecular mechanisms underlying self-renewal in stem cells. In hematopoietic stem cells (HSCs), the best-studied mammalian stem cells, self-renewal has been demonstrated by *in vivo* assays (1–4). However, molecular mechanisms regulating self-renewal remain poorly understood. In particular, despite numerous studies of cytokines and cytokine receptors, little is known about intracellular signaling events in self-renewal of HSCs (5–7). Major difficulties have been the paucity of HSCs and the *in vitro* recapitulation of self-renewal (8, 9). We have approached this issue by analyzing Lnk-deficient mice (Lnk−/−) in comparison with WT mice.

Lnk is an adaptor protein containing a proline-rich domain, a pleckstrin homology domain, and a Src homology 2 domain (10). In Lnk−/− mice, long-term marrow repopulating activity is markedly elevated because of increases in both absolute number and self-renewal activity of HSCs (4, 11). These results suggest that Lnk negatively regulates the key signaling pathways of HSC self-renewal. Lnk is expressed in various hematopoietic lineages, in which some of its functions have been reported (12–15). Lnk is thought to regulate stem cell factor (SCF) signaling pathways negatively in immature B cells (12, 13). Recent reports indicated that Lnk negatively regulates thrombopoietin (TPO) signaling in megakaryocytes and erythropoietin signaling in erythroblasts (14, 15). Although the functions of Lnk as a negative regulator of cytokine signaling are shared by these lineages, the target signaling pathways appear to differ among these lineages. We therefore attempted to determine Lnk target signaling pathways in HSCs.

In both WT and Lnk−/− mice, CD34-negative or low, c-Kit-positive, Sca-1-positive, lineage marker-negative ($CD34^-KSL$) cells within adult mouse bone marrow (BM) are highly enriched in HSCs (4, 16). When single-cell transplantation with $CD34^-KSL$ cells was performed, rates of long-term reconstitution were similar in WT and Lnk −/− mice, indicating similar degrees of HSC enrichment in this population. Using these highly enriched HSC populations, we first studied cytokine-induced division of $CD34^-KSL$ cells and found that Lnk is involved in the TPO signaling pathway. We then investigated how HSCs self-renew in culture with TPO by competitive repopulation and paired daughter cell assays. Furthermore, we developed single-cell immunostaining procedures for signal transduction analysis to examine Lnk-interacting intracellular signaling pathways in TPO-stimulated $CD34^-KSL$ cells.

Results

***In Vitro* Survival and Division of Single $CD34^-KSL$ Cells.** Direct effects of cytokines on both survival and proliferation of HSCs were evaluated to see which cytokine signals are influenced by the absence of Lnk. Serum-free culture of single WT or Lnk−/− $CD34^-KSL$ cells was performed in the presence of various cytokines at 100 ng/ml. Every 24 h after initiation of culture, cells in each well were examined under the microscope. At 72 h of culture, no $CD34^-KSL$ cells survived without a cytokine. In contrast, >70% of cells survived in the presence of SCF or TPO, and <20% of cells survived in the presence of IL-3, IL-6, or IL-11 (Fig. 1*A*). When cells were cultured with SCF, frequencies of cell division did not differ between Lnk−/− $CD34^-KSL$ cells (47.9 ± 5.3%) and WT $CD34^-KSL$ cells (54.6 ± 9.1%) (P =

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The authors declare no conflict of interest.

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Abbreviations: HSC, hematopoietic stem cell; SCF, stem cell factor; TPO, thrombopoietin; BM, bone marrow; RU, repopulating unit; SCIPhos, single-cell imaging of phosphorylation

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Rheumatoid Arthritis Fibroblast-like Synoviocytes Express BCMA and Are Stimulated by APRIL

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Objective. **Fibroblast-like synoviocytes (FLS) are among the principal effector cells in the pathogenesis of rheumatoid arthritis (RA). This study was undertaken to examine the variety of stimulating effects of APRIL and its specific effect on FLS in the affected RA synovium.**

Methods. **Synovium and serum samples were obtained from patients with RA, patients with osteoarthritis (OA), and healthy subjects. Soluble APRIL proteins were assayed by enzyme-linked immunosorbent assay. The relative gene expression of APRIL, BCMA, interleukin-6 (IL-6), tumor necrosis factor α (TNFα), IL-1β, and RANKL was assessed in RA and OA FLS by polymerase chain reaction. Effects of APRIL on the production of proinflammatory cytokines and RANKL in RA FLS were investigated by flow cytometry and with the use of a BCMA-Fc fusion protein.**

Results. **A significantly higher level of soluble APRIL was detected in RA serum compared with normal serum. Among the 3 receptors of APRIL tested, RA FLS expressed only BCMA, whereas OA FLS expressed none of the receptors. APRIL stimulated RA FLS, but not OA FLS, to produce IL-6, TNFα, IL-1β, and APRIL itself. In addition, APRIL increased RA FLS expression of RANKL and also enhanced progression of the cell cycle of RA FLS. Neutralization of APRIL by the BCMA-Fc fusion protein attenuated all of these stimulating effects of APRIL on RA FLS.**

Conclusion. **RA FLS are stimulated by APRIL and express the APRIL receptor BCMA. These results provide evidence that APRIL is one of the main regulators in the pathogenesis of RA.**

Rheumatoid arthritis (RA) is characterized by joint destruction resulting from chronic inflammation in the synovial tissue. The chronicity of the disease is postulated to be maintained by interactions between infiltrating mononuclear cells and synovial cells (1,2), in addition to the autocrine stimulatory effects of proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 (1). Fibroblast-like synoviocytes (FLS) act as one of the main effector cells in the joint destruction of RA, through their ability to invade and degrade soft tissue and cartilage (1,3). FLS can also stimulate the differentiation and activation of osteoclasts, resulting in bone erosion (4–6).

Recent research has provided important information about the signaling mechanisms that can target FLS in the affected RA synovium, such as mediators of inflammation, cytokines, and cell–cell and cell–extracellular matrix interactions (7). These signaling mechanisms underlie the ability of RA FLS to drive migration, proliferation, and matrix degradation. Moreover, RA FLS have been shown to proliferate in an anchorage-independent manner, to lack contact inhibition, and to constitutively express cytokines, oncogenes, and cell cycle proteins, in a transformation-related manner (8,9).

BAFF (also termed B lymphocyte stimulator, or BlyS [trademark of Human Genome Sciences, Rockville, MD]), a member of the TNF family, is essential for B cell generation, maintenance, and autoreactivity (10–12). High levels of BAFF are detectable in the sera of patients with autoimmune rheumatic diseases, particularly systemic lupus erythematosus (SLE) and Sjögren's syndrome (13–15). BAFF is also present at high levels in

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Research article

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Association of the diplotype configuration at the *N*-acetyltransferase 2 gene with adverse events with co-trimoxazole in Japanese patients with systemic lupus erythematosus

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Abstract

Although co-trimoxazole (trimethoprim-sulphamethoxazole) is an effective drug for prophylaxis against and treatment of *Pneumocystis* pneumonia, patients often experience adverse events with this combination, even at prophylactic doses. With the aim being to achieve individual optimization of co-trimoxazole therapy in patients with systemic lupus erythematosus (SLE), we investigated genetic polymorphisms in the *NAT2* gene (which encodes the metabolizing enzyme of sulphamethoxazole). Of 166 patients with SLE, 54 patients who were hospitalized and who received prophylactic doses of co-trimoxazole were included in the cohort study. Adverse events occurred in 18 patients; only two experienced severe adverse events that lead to discontinuation of the drug. These two patients and three additional ones with severe adverse events (from other institutions) were added to form a cohort sample and were analyzed in a case-control study. Genotype was determined using TaqMan methods, and haplotype was inferred using the maximum-likelihood method. In the cohort study, adverse events occurred more frequently in those without the *NAT2*4* haplotype (5/7 [71.4%]) than in those with at least one *NAT2*4* haplotype (13/47 [27.7%]; $P = 0.034$; relative risk = 2.58, 95% confidence interval = 1.34–4.99). In the case-control study the proportion of patients without *NAT2*4* was significantly higher among those with severe adverse events (3/5 [60%]) than those without severe adverse events (6/52 [11.5%]; $P = 0.024$; odds ratio = 11.5, 95% confidence interval = 1.59–73.39). We conclude that lack of *NAT2*4* haplotype is associated with adverse events with co-trimoxazole in Japanese patients with SLE.

Introduction

Co-trimoxazole (trimethoprim-sulphamethoxazole) is an effective drug in the prevention and treatment of *Pneumocystis* pneumonia [1,2], a life-threatening condition that mainly occurs in immunodeficient patients. Usage of the drug was recently extended to patients with connective tissue disease, including systemic lupus erythematosus (SLE) [3]. Although co-trimoxazole was confirmed to have prophylactic effect against *Pneumocystis* pneumonia in SLE patients, it often causes adverse events, even at prophylactic doses. Adverse events include life-threatening conditions such as toxic epidermal necrolysis (TEN) and Stevens-Johnson syndrome (SJS), hepatotoxicity, haematological toxicity and gastrointestinal manifestations [4].

ALT = alanine aminotransferase; AST = aspartate aminotransferase; GST = glutathione *S*-transferase; NAT2 = *N*-acetyltransferase 2; PM/DM = polymyositis/dermatomyositis; SLE = systemic lupus erythematosus; SNP = single nucleotide polymorphism; SJS = Stevens-Johnson syndrome; TEN = toxic epidermal necrolysis.

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Importance of the Hydrogen Bonding Network Including Asp52 for Catalysis, as Revealed by Asn59 Mutant Hen Egg-white Lysozymes

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In the catalysis of sugar hydrolysis by hen egg-white lysozyme, Asp52 is thought to stabilize the reaction intermediate. This residue is involved in the well-ordered hydrogen bonding network including Asn46, Asp48, Ser50 and Asn59 on the anti-parallel β-sheet, designated as a 'platform', on which the substrate sugar sits. To reveal the role of this hydrogen bonding network in the hydrolysis, we characterized Asn59 mutants by biochemical and crystallographic studies. Surprisingly, the introduction of only a methylene group by the Asn59Gln mutation markedly reduced the bacteriolytic activity and abolished the hydrolytic activity towards the synthetic substrate, PNP-$(GlcNAc)_5$. A similar result was also obtained with the Asn59Asp mutant. The crystal structure of the Asn59Asp mutant in complex with the substrate analogue revealed that, as in the wild-type, the $(GlcNAc)_3$ was bound in the A–B–C subsites. The reduced activity would be caused by subtle changes in the side-chain orientations as well as the electrostatic characteristics of Asp59, resulting in the rearrangement of the hydrogen bonding network of the platform. These results suggest that the precise locations of these 'platform' residues, maintained by the well-ordered hydrogen bonding network, are crucial for efficient hydrolysis.

Key words: catalytic activity, lysozyme, protein–carbohydrate interactions, protein engineering, X-ray crystallography.

Abbreviations: PNP, *p*-nitrophenyl; GlcNAc, N-acetyl-β-D-glucosaminine (PNP-GlcNAc); MurNAc, N-acetylmuramic acid; SDS, sodium dodecyl sulfate; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance.

Hen egg-white lysozyme catalyses the hydrolysis of β-1,4 glycosidic bonds of alternating copolymers of GlcNAc and MurNAc[1] in bacterial cell walls as well as the homopolymer of GlcNAc, chitin (*1–3*). Hen egg-white lysozyme has been extensively studied by functional and structural analyses (*4*), and has six binding subsites for the sugar residues, termed A–F (*5*, *6*). Catalytic models of sugar hydrolysis by hen egg-white lysozyme were proposed independently by Phillips (*7*) and Koshland (*8*). The first step is well-understood, and involves Glu35, which possesses an unusual pKa, protonating the oxygen connecting the sugar residues D and E. On the other hand, in the next step, the role of Asp52, which was proposed to stabilize the oxocarbonium ion of the intermediate state in the hydrolysis, still remains unclear. Kuroki *et al.* (*9*) reported the crystal structure of the mutant Asp52Glu complexed with $(GlcNAc)_6$, which clearly revealed the covalent bond between the Oε atom of Glu52 and the C1 atom of sugar D of the resultant hydrolysis product $(GlcNAc)_4$. However, it seems unlikely that the wild-type forms the covalent bond during catalysis, because Asp52 is still too far from the C1 of sugar D. Moreover, Vocaldo *et al.* (*10*) detected the covalent intermediate of the Glu35Gln mutant lysozyme with $(GlcNAc)_2GlcF$ through Asp52. On the basis of a comparison with other β-glycosidases, they proposed that the anomer-retaining β-glycosidases adopt the general catalytic mechanism, including the formation of the covalent intermediate.

Although Asp52 has a normal pKa, it participates in the well-ordered hydrogen bonding network called the 'platform' (*5*), which includes Asn46, Asp48, Ser50 and Asn59. As expected, several previous studies showed that the replacement of Asp52 reduced the hydrolytic activity to <5% of the wild-type. However, except for the studies of the Asn46 mutant, which also showed a reduction of the hydrolytic activity (*11*), only limited information is available about the role of this hydrogen bonding network in the hydrolysis. To clarify the functional and structural relevance of the platform hydrogen bonding network on the activity, we focused on the residue Asn59, which directly interacts with the catalytic residue Asp52, and analysed the mutants Asn59Asp and Asn59Gln by biochemical and crystallographic methods.

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