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Characterization of protein O-GlcNAc in neutrophils.

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CHARACTERIZATION OF PROTEIN *O*-GlcNAc IN NEUTROPHILS

by

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ABSTRACT OF DISSERTATION
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Title Characterization of Protein *O*-GlcNAc in Neutrophils

A variety of cytoplasmic and nuclear proteins can be modified on serine and threonine residues by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc), although the effects of this modification on protein and cellular functions are not completely defined. Here, the physiological effects of the *O*-GlcNAc modification are examined in the context of receptor-mediated signal transduction, with an emphasis on cellular motility and motility-associated signaling intermediates. We demonstrate for the first time that protein *O*-GlcNAc can be induced by ligand engagement of cognate cell surface receptors, and that the temporal dynamics of this process can be very rapid. We also show that neutrophils possess a functional hexosamine biosynthesis pathway that in response to exogenous stimulation produces UDP-*N*-acetylglucosamine sufficient to drive *O*-GlcNAcylation and enhance agonist-induced *O*-GlcNAc responses. By metabolic and pharmacological manipulation of the enzymes of *O*-GlcNAc cycling, we demonstrate that increased *O*-GlcNAcylation enhances both basal and chemoattractant-directed cellular motility. We show that this enhancement is due to *O*-GlcNAc-associated changes in the activities of several key motility-associated signaling intermediates. These studies suggest that *O*-GlcNAcylation is a highly dynamic signal that can be induced by agonist to modulate the activities of signaling elements involved in the regulation of cellular motility.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
Ca^{2+}	calcium
DMEM	dulbecco's modification of Eagle's medium
ERK	extracellular-regulated kinase
fMLF	formylated-methionine-leucine-phenylalanine
$GalNH_2$	galactosamine
GEF	guanine nucleotide exchange factor
GlcNAc	*N*-acetylglucosamine
$GlcNH_2$	glucosamine
$GlcNH_2$ 6-P	glucosamine 6-phosphate
HBP	hexosamine biosynthesis pathway
HBSS	hank's buffered salt solution
HPLC	high performance liquid chromatography
HSP27	heat shock protein 27
LSP1	leukocyte-specific protein 1
MAPK	mitogen-activated protein kinase
MAPKAPK	mitogen-activated protein kinase-activated protein kinase
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
O-GlcNAc	*O*-linked β-*N*-acetylglucosamine

O-GlcNAcase	neutral β-*N*-acetylglucosaminidase
OGT	UDP-GlcNAc: polypeptide *O*-β-*N*-acetylglucosaminyltransferase
OPA	*O*-pthalaldehyde
PAK-1 PBD	p21-binding domain of p21-activated kinase 1
PBS	phosphate buffered saline
Phox	phagocyte oxidase
PI3K	phosphoinositide-3 kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMN	neutrophil or polymorphonuclear leukocyte
PugNAc	*O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate
Raf-1 RBD	Ras-binding domain of Raf-1
ROS	reactive oxygen species
UDP-GlcNAc	UDP-*N*-acetylglucosamine

INTRODUCTION

Neutrophil Function in Innate Immunity

In the late 19th century, after observing the activities of amoeboid cells in starfish larvae, Élie Metchnikoff developed the theory of phagocytosis, which held that specialized cells are able to engulf and destroy harmful intruders such as bacteria (1). His observations and the theory that developed from it eventually led Metchnikoff to a definition of immunity as a complex system of defensive mechanisms evolved to promptly detect and kill infectious intruders. These protective responses can be divided into two components: innate and adaptive. Innate resistance is functional without previous exposure to an infectious agent and is non-specific, using the same basic neutralizing mechanisms (for example, phagocytosis) for different pathogens. The defining feature of adaptive resistance, on the other hand, is its specific, inducible response to pathogens. Innate immunity is composed of physical barriers such as skin and mucous membranes, secreted products such as lytic enzymes and the complement system, and specialized bloods cells (white blood cells or leukocytes) such as neutrophils and macrophages. These specialized cells not only are the primary players in innate immunity but also are responsible for developing acquired responses to infection (2).

Neutrophils are the most abundant cellular mediators of innate immunity. They derive from myeloid progenitors in the bone marrow and belong to the granulocyte family of white blood cells. As such, neutrophils are ontologically related to eosinophils, basophils, mast cells, and macrophages. They are characterized by multilobed nuclei

(hence the name polymorphonuclear leukocytes or PMNs) and densely staining cytoplasmic granules that contain host-defense factors (3).

The importance of neutrophils can best be appreciated by examining the effects of inherited disorders on neutrophil function. For example, individuals with chronic granulomatous disease lack the ability to produce the toxic oxygen species required to kill pathogens (4). Because of this lack, they suffer from chronic recurrent bacterial and fungal infections that are not effectively contained and are likely to spread systemically from their original location. The capacity for pathogen killing also carries with it an implicit capacity for host tissue destruction; as such neutrophils may also participate in inflammatory and autoimmune disease (5).

Neutrophil Functional Outputs

The response to infection or tissue damage involves the sequential recruitment of various immune cells to a site of injury. Neutrophils are the first cell type to arrive during the course of inflammation, usually appearing at a site of infection within hours. They are recruited from the circulation and triggered to extravasate into locally infected peripheral tissues by various chemotactic factors. Offending pathogens are subsequently eliminated by phagocytosis and the release of antimicrobial oxygen metabolites, proteases, and peptides (6, 7).

Rolling, adherence, and extravasation. The extravasation of circulating neutrophils requires that they roll on and form transient adhesive contacts with activated endothelial cells. Rolling is mediated by selectins and tissue-specific mucin-like glycoproteins

containing sialyl-Lewis X moieties that bind selectin lectin domains (8, 9). The cells then form tight, stationary adhesive complexes through integrins and immunoglobulin-like adhesive molecules such as cell adhesion molecule and intercellular cell adhesion molecule (10, 11), and transmigrate through the endothelium (12, 13).

Motility and chemotaxis. The presence of an infection not only induces neighboring endothelial cells to promote extravasation but also results in the localized appearance of chemotactic factors that guide neutrophils through the surrounding matrix to a specific site of infection. These chemotactic signals include activated serum proteins (C5a) (14), interleukin 8 (15), matrix metalloproteinases (16), and the products of invading microorganisms (formylated peptides) (17). When exposed to a chemotactic gradient, neutrophils rapidly orient themselves and move through amoeboid motion using anterior pseudopod extension together with posterior contraction and retraction (18).

Phagocytosis and respiratory burst. Once at a site of infection, neutrophils must recognize and eliminate offending pathogens. Although neutrophils are able to directly recognize the surface components of microbes, recognition is greatly enhanced if the microbes are first opsonized with serum proteins such as complement and antibody (19). This recognition leads to the initiation of phagocytosis and the concurrent initiation of a killing program that culminates in the release of a variety of cytotoxic agents into the developing phagosome. These cytotoxic agents can be classified as oxygendependent or as oxygenindependent (20). The oxygenindependent group is contained within the three neutrophil granule subsets (the azurophilic, specific, and gelatinase granules) and is com-

posed of proteases, antimicrobial proteins and peptides, and enzymes. The second group of cytotoxic agents is oxygendependent. Its constituents, reactive oxygen species (ROS), are the products of an oxidative or respiratory burst generated by the activity of a membrane-associated nicotine adenine dinucleotide phosphate (NADPH) oxidase complex. The oxidase complex assembles at phagosome membranes and produces superoxide anions (21) that are rapidly converted to hydrogen peroxide by the enzyme superoxide dismutase (22). Myeloperoxidase, a component of azurophilic granules, generates hypochlorous acid from hydrogen peroxide (23). The mechanisms through which superoxide and its byproducts cause microbial killing are unclear (20). However, the respiratory burst that generates these oxidative intermediates is essential for pathogen elimination, as illustrated by the susceptibility to infections of patients suffering from chronic granulomatous disease, a condition defined by a lack of NADPH oxidase activity (4).

The Respiratory Burst

The NADPH Oxidase Complex

In resting cells, the NADPH oxidase consists of unassembled cytosolic and membrane components. The membranes of secretory vesicles and specific granules contain flavocytochrome b_{558}, a dimer composed of $p22^{phox}$ and $gp91^{phox}$ subunits (*phox* refers to *ph*agocyte *ox*idase), and an associated low molecular weight G protein, Rap1. The cytosolic component consists of a complex of three proteins, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. During stimulation this trimer, particularly the $p47^{phox}$ subunit, undergoes extensive phosphorylation and binds Rac-GTP. This allows it to migrate to and associate with cyto-

chrome b_{558} for the assembly of an active oxidase that catalyzes the transfer of an electron from NADPH to molecular oxygen to form ROS (24).

Signaling the Respiratory Burst

Oxidase activity is tightly regulated by protein kinases, lipid kinases, and small G proteins. These signaling intermediates can be activated by a large and diverse group of stimuli. Formylated peptide chemoattractants such as the tripeptide formylated-methionine-leucine-phenylalanine (fMLF) are robust inducers of the NADPH oxidase and have been used extensively in the study of the signaling pathways that regulate its activity. The respiratory burst initiated by fMLF begins with the engagement of cellsurface heterotrimeric G protein-coupled formylated peptide receptors and the dissociation of trimeric G protein complexes into G_{α} and $G_{\beta\gamma}$ subunits (25).

Calcium, phospholipase C (PLC) and protein kinase C (PKC). Receptor engagement is closely followed by a transient increase in cytosolic calcium (Ca^{2+}) resulting from both the release of Ca^{2+} from internal stores, mediated by inositol 1,4,5-triphosphate and PLC, and extracellular Ca^{2+} influx (26, 27). Blocking cytosolic Ca^{2+} transients with intracellular Ca^{2+} chelators inhibits the respiratory burst (28). Double knockout mice for PLCβ2 and PLCβ3 are completely unable to generate inositol 1,4,5-triphosphate or evoke Ca^{2+} transients and display significant deficiencies in ROS production in response to fMLF (29). With respect to oxidase components, the activation of the small G protein Rac does not appear to be affected in the PLCβ2/3 double knockout, but $p47^{phox}$ phosphorylation is inhibited (29).

PLC activity results in diacylglycerol production, which activates PKC in conjunction with Ca^{2+} (30). Phorbol myristate acetate, a diacylglycerol analogue, is a potent activator of ROS formation (31); the PKC inhibitors staurosporine and Ro 31-7549 are potent inhibitors (32) of ROS formation. Studies have shown that $p47^{phox}$ is a substrate for PKC and that PKC-phosphorylated $p47^{phox}$ activates the NADPH oxidase (33).

Mitogen-activated protein kinases (MAPKs). MAPK family members also appear to control NADPH oxidase activity. fMLF activates both p38 MAPK and the extracellular signal-regulated kinases (ERKs), p42 and p44 (34). MAPK kinase 3 appears to activate p38, whereas the MAPK kinases MAPK ERK kinase 1 and 2 (MEK1/2) activate p42/44 (34, 35). Upstream regulation of MEK1/MEK2 may occur through the MAPK kinase Raf as it is strongly activated during fMLF stimulation (35). Both PP1, an inhibitor of Src-family kinases, and a $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple mutation (Lyn, Fgr, and Hck are Src-family kinases) inhibit p38 phosphorylation in murine neutrophils (36). The situation is similar for $rac1^{-/-}$ and $rac2^{-/-}$ knockouts, with inhibition of both p42/44 and p38 phosphorylation (37, 38). The inhibition of either p38 or ERK blocks agonist-induced ROS formation (34, 35). p44/42 and p38 are capable of phosphorlyating $p47^{phox}$, although p38 activity alone is not sufficient for NADPH oxidase activation (39). MEK1/MEK2 are activated by PKC agonists and conversely inhibited by PKC antagonists, indicating that PKC is an upstream mediator of ERK activity in neutrophils (40). Studies have also found that PLC activity is required for optimal fMLF-evoked activation of p38 MAPK and ERK (29). In addition to PKC, the Ca^{2+}-sensitive intermediate calmodulin may mediate MAPK activity. In neutrophils Ca^{2+}-sensitive intermediate calmodulin has been

linked to the activation of Ras and Rac, which are upstream regulators of the MAPK cascade (36, 41, 42).

Phosphatidylinositol-3 kinase (PI3K) and phosphatidylinositol 3,4,5-trisphosphate (PIP3). Knockout studies in mice have revealed the importance of phosphoinositide metabolism in chemoattractant-induced ROS formation (29). In contrast to neutrophils from wild-type mice, the neutrophils from mice lacking PI3K γ are unable to produce PIP3 in response to fMLF and are unable to generate ROS (29). Ca^{2+} mobilization, PKC activation, and $p47^{phox}$ translocation do not appear to be affected in these PI3Kγ knockouts. Basal and fMLF-stimulated increases in PI3K activity are resistant to changes in intracellular Ca^{2+} and the PKC antagonist staurosporine (43). Interestingly, inhibitors of PI3K, such as wortmannin, block fMLF-induced ERK and p38 MAPK activation, indicating that PI3K may lie upstream of the MAPK pathways in NADPH oxidase activation (41).

Cellular Motility and Migration

Neutrophil Chemotaxis

The term *chemotaxis* was conceived by Pfeffer in 1884 to describe the attraction of fern sperm to ova (44) and is now used to describe the migration of bacteria and eukaryotic cells along chemical concentration gradients. The chemical attractants that signal chemotaxis are called chemoattractants. Neutrophils depend on directed migration and motility to home in on and eliminate pathogens (3). They migrate toward chemoattractant sources such as bacterial formylated peptides; C5a, a product of the complement cascade;

products of phospholipid metabolism (leukotriene B_4); and chemotactic cytokines (chemokines) such as interleukin 8 (3). The chemoattractant fMLF has been used extensively as a tool to study neutrophil migration (17). The stimulation of formyl peptide receptors results in the activation of a signaling cascade that directs changes in the actin-based cytoskeletal machinery. These cytoskeletal changes provide for the mechanical means of producing movement and culminate in a polarized morphology of leading edge pseudopod extension and posterior uropod contraction and retraction. The actin filaments within these regions are collected into highly organized structures that are regulated by a variety of actin-binding proteins that behave to push the membrane forward or pull it backward (45).

Signaling Neutrophil Motility

PI3K and PIP3. The engagement of formyl peptide receptor results in the release of G protein α and $\beta\gamma$ subunits that are then free to interact with downstream effectors such as PLC and PI3K, respectively (25). Data obtained from PLC-deficient mice indicate that, in contrast to the respiratory burst, PLC and associated Ca^{2+} transients are not required for neutrophil chemotaxis in response to fMLF (29). The principal PI3K of fMLF-associated signaling, PI3Kγ, is directly activated by $G_{\beta\gamma}$ to produce PIP3 (46). A variety of pleckstrin homology domain-containing proteins can bind PIP3 (47). PIP3 accumulates at the leading edge of migrating cells, the site of maximal actin polymerization in activated neutrophils, and is thought to generate a steep internal signaling gradient that attracts the signaling elements responsible for organizing actin-associated motility (48).

Disrupting PIP3 production by either inhibiting PI3K lipid kinase activity with wortmannin or knocking out the PI3K gene in mice results in impaired motility (49-52).

A primary means of polarizing neutrophils in response to an fMLF gradient is the selective accumulation of PIP3 at the leading edge of cells. This is accomplished in part by decreased phosphatase and tensin homologue-mediated PIP3 degradation at the front of cells. In polarized cells phosphatase and tensin homologue is localized to the uropod, allowing leading edge PIP3 accumulation. This accumulation is likely also directed by a global inhibitory process that offsets heterotrimeric G protein activation at the back of the cell and thereby localizes responses to the front (48).

The low molecular weight GTPase Rac. Members of the Ras GTPase superfamily related to Rho, in particular Rac, are also important regulators of motility and cell shape. These GTPases alternate between active GTP-bound and inactive GDP-bound states in a cycle regulated by guanosine dissociation inhibitors, GTPase-activating proteins, and guanine exchange factors (GEFs) (53). A variety of studies have now confirmed that Rac plays a critical role in neutrophil motility. Most significantly, Rac1- and Rac2 -deficient murine neutrophils display severe deficiencies in fMLF-induced polarity and chemotaxis (37, 54-56). Although three isoforms of Rac have been identified, most studies have focused on the roles of Rac1 and Rac2 (57). Rac1 is ubiquitously expressed, whereas Rac2 expression is restricted to hematopoietic cells (57). The distinct roles of these isoforms in chemotaxis and motility are largely undefined; however recent evidence suggests that Rac2 may be the primary regulator of actin assembly and the motile machinery, whereas Rac1 may be more important for polarization and gradient detection (58). Rac activity

may be regulated in large part by a recently identified GEF, P-Rex1 (59). This Rac GEF is activated by either phosphatidylinositol phosphates or $G_{\beta\gamma}$, thus providing a direct link between PI3K and Rac. The PI3K inhibitor wortmannin has also been found to block fMLF-induced Rac activation (60). In PI3K-deficient mice, however, fMLF still induces Rac-GTP (29), indicating that other pathways lead to Rac activation (accordingly, the PIP3-associated GEF P-Rex1 accounts for only 60% of neutrophil Rac GEF activity (59)).

MAPKs. Rac-deficient murine neutrophils also display impaired fMLF-induced p38 and ERK activation (37, 38, 54). This is significant because pharmacological MAPK inhibition has indicated that these MAPKs are important mediators of chemotactic signaling (61-68). It is not clear how they are involved, although both p38 and ERK phosphorylate and activate Mitogen activated protein kinase-activated protein kinase (MAPKAPK) (68, 69). MAPKAPK knockout mice display deficiencies in neutrophil motility (68, 70, 71). MAPKAPK is involved in the regulation of two potentially important downstream intermediates with ties to the actin cytoskeleton: heat shock protein 27 (72) and leukocyte-specific protein 1 (73). Leukocyte-specific protein 1 is an actin-binding protein that modulates cell motility and is a major target of MAPKAPK kinase activity (73-75). MAPKAPK also phosphorylates heat shock protein 27, and heat shock protein 27 has been shown to modulate actin polymerization and migration in a variety of cell types (72, 76-78).

Regulation of the actin cytoskeleton. Chemoattractant-induced actin polymerization is restricted to the edges of developing pseudopods and is concentrated at the tips of actin bundles that project into the plasma membrane (79). The Arp2/3 complex is a central mediator of this polymerization (80). The Arp2/3 complex localizes to sites of actin polymerization such that it is distributed to surfaces that receive maximal chemotactic stimulation (81). It promotes de novo actin nucleation and actin polymerization (80). Rac regulates Arp2/3 activity through Wiskott-Aldrich syndrome protein (82). Gelsolin, an actin filament-capping protein, is another motility-associated effector that is regulated by Rac (83). Rac induces the dissociation of gelsolin from the barbed ends of actin filaments allowing for the initiation of actin polymerization in response to chemoattractants.

Protein *O*-linked β-*N*-acetylglucosamine

Post-translational Protein Modifications

A multitude of dynamic molecular signaling events govern the basic biological functions of growth, reproduction, and responses to the environment. Proteins are a principal means of mediating these processes through their dynamic interaction with each other and with metabolites, phospholipids, carbohydrates, and nucleic acids. Protein activity is controlled by biosynthesis, degradation, and selective covalent processing, which is termed post-translational modification and modulates protein catalytic activity, molecular interactions, localization, and stability. Post-translational modifications are essential in the dynamic modulation of cellular function. They are catalyzed by substrate-specific enzymes and are themselves under strict control by post-translational modification. Proteins can be present in several differentially modified forms derived, for exam-

ple, from splice variants to multiple phosphorylated, glycosylated, or acylated states, which all give rise to substantial heterogeneity in the protein population (often referred to as the proteome, the study of which is called proteomics).

O-linked Glycosylation of Nuclear and Cytoplasmic Proteins

The addition of single *O*-glycosidic *N*-acetylglucosamine subunits in β linkage to Ser/Thr residues (*O*-GlcNAc) is a unique form of protein glycosylation that occurs on numerous cytoplasmic and nuclear proteins and is distinct from the complex form of glycosylation that occurs on proteins synthesized in the secretory pathway (84, 85). Numerous intracellular proteins appear to be modified by this post-translational modification. They include nuclear pore components (85, 86), a variety of transcription factors (87, 88), cytoskeleton-associated proteins, and signaling enzymes (89, 90).

Early work on *O*-GlcNAcylation suggested that it was dynamic and inducible (91-93). This finding led to the prediction that *O*-GlcNAcylation would have a regulatory role analogous to phosphorylation. In fact, because these post-translational modifications appeared to share common target residues, serines and threonines, it was proposed that they might have a "yin-and-yang" relationship (91). There is now a significant and growing pool of evidence in support of this under certain conditions (94). The cloning and characterization of enzymes responsible for the addition, UDP-GlcNAc: polypeptide *O*-β-*N*-acetylglucosaminyltransferase (OGT), and removal, neutral β-*N*-acetylglucosaminidase, of *O*-GlcNAc moieties have provided support for the idea that this modification is regulated and may be dynamic (95-97). Although each of these enzymes is encoded by a single gene, as opposed to the great variety of genes and associated gene products that are

associated with kinase activity, they are potentially regulated through a variety of mechanisms that could allow a heterogeneous, context-dependent control of *O*-GlcNAcylation: alternative splicing, proteolytic processing, association with a variety of cofactors, post-translational modification, regulation by substrate availability, and multimerization (97). Enzyme specificity and activity could be influenced by any of these factors. Over the last few years a significant body of research has accumulated as a result of the effort to unravel the functional roles of *O*-GlcNAc and the mechanisms through which it may modulate cellular signaling pathways, and thereby cellular function, in health and disease (94). For example, altered *O*-GlcNAcylation has been extensively investigated with respect to its role in the pathogenesis of diabetes mellitus (89, 98-100). It has also become apparent that it may play a role in the etiology of certain cancers (101, 102) and Alzheimer disease (103, 104).

The hexosamine biosynthesis pathway (HBP). HBP is an essential component of the pathway leading to *O*-GlcNAcylation. In fact, many of the approaches used to investigate *O*-GlcNAc function rely on the manipulation of hexosamine biosynthesis. It is thought that under normal conditions ~ 2-5% of the total glucose transported into cells feeds into the HBP (105). This begins at the level of fructose 6-phosphate, which may either pass into glycolysis, the hexose monophosphate shunt or the HBP. The HBP is entered through the activity of L-glutamine:D-fructose 6-phosphate amidotransferase, which catalyzes the formation of glucosamine ($GlcNH_2$) 6-phosphate ($GlcNH_2$ 6-P) from fructose 6-phosphate (106). This enzyme is the rate-limiting intermediate in hexosamine biosynthesis. Within the HBP, $GlcNH_2$ 6-P is metabolized through various intermediates

to UDP-*N*-acetylglucosamine (UDP-GlcNAc), the sugar donor in complex secretory glycoprotein formation and OGT-mediated *O*-GlcNAcylation (105). A key feature in the regulation of OGT is that its activity is sensitive to relatively small changes in substrate availability over a wide range of substrate concentrations; additionally, it recognizes different acceptor proteins at different concentrations of UDP-GlcNAc (107). Thus, as several studies have shown, when cells in culture or tissues *in vivo* are exposed to hyperglycemic conditions, they exhibit enhanced levels of *O*-GlcNAc that can be blocked by HBP inhibitors such as azaserine (87, 98, 108). Specifically, elevated levels of glucose lead to enhanced HBP flux and UDP-GlcNAc formation. Elevated levels of UDP-GlcNAc in turn drive protein *O*-GlcNAcylation in an OGT-dependent manner (107). Exogenous $GlcNH_2$, which is not normally present in the cellular environment at appreciable levels, is also transported into cells and preferentially metabolized through this pathway (89, 105, 108). The resulting UDP-GlcNAc also drives OGT activity and leads to enhanced protein *O*-GlcNAc.

O-*GlcNAcylation* versus *phosphorylation.* Protein *O*-GlcNAc is thought to regulate critical aspects of protein biology, including protein stability, subcellular localization,and protein-protein interactions (94). Thus in many respects it would seem to be analogous to phosphorylation, the archetypal regulatory mechanism for the coupling of extracellular signals to specific cell responses, in terms of its function.

Much of the work on the functional significance of *O*-GlcNAc has relied on long-term treatments, hours or days, with either high glucose (87, 98, 99) or $GlcNH_2$ (89, 108). A small handful of pharmacological agents have also been found to inhibit specific en-

zymes in the pathways affecting *O*-GlcNAcylation. These reagents include *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (PugNAc) and alloxan, which increase and decrease *O*-GlcNAc through the inhibition of neutral β-*N*-acetylglucosaminidase and OGT, respectively (109, 110). Lymphocytes provided early evidence that *O*-GlcNAcylation can be a dynamic process, as pharmacological mitogens were shown to induce changes in cellular *O*-GlcNAc over times as short as 1 h (92). In addition, certain pharmacological agents that bypass physiologically relevant agonist- and receptor-specific signaling mechanisms induce relatively rapid changes in *O*-GlcNAc. In particular, pharmacological treatments with the calcium ionophore A23187 and the protein phosphatase inhibitor okadaic acid have been shown to induce changes in *O*-GlcNAc within 1 min, suggesting that *O*-GlcNAc responses may indeed proceed quite rapidly (111). These examples are often used as corroborative evidence of the *O*-GlcNAc potential as a signaling mechanism that is analogous to phosphorylation.

The physiological role(s) of O-*GlcNAcylation.* Although protein *O*-GlcNAcylation is emerging as an important post-translational modification that regulates protein function, the physiological contexts in which changes in the level of *O*-GlcNAc occur are only beginning to be elucidated. Changes in *O*-GlcNAc are strongly implicated in the pathogenesis of diabetes mellitus (89, 98-100), a disease characterized by chronic hyperglycemia, and have also been suggested to serve as a nutrient sensor, in which *O*-GlcNAc signals are modulated through nutrient availability (112). Recent work has also suggested that *O*-GlcNAc may couple proteasomes to the general metabolic state of the

cell (113). In addition, *O*-GlcNAc appears to modulate stress-activated signaling networks and may be integral to cellular survival responses (114).

Objective of Dissertation

There is only limited evidence of agonist-induced, cellsurface receptor-mediated changes in *O*-GlcNAcylation. Although the infusion of insulin for several hours leads to increased protein *O*-GlcNAc, this response is most likely due to increased glucose transport and a slowly progressive increase in HBP flux rather than to a direct effect of insulin on the *O*-GlcNAcylation machinery (89, 115, 116). In addition, there is little evidence to suggest that, with respect to the temporal dynamics, *O*-GlcNAcylation can occur rapidly. An essential characteristic of many signal transduction mechanisms, exemplified by protein phosphorylation, is that they are activated by agonist in a rapidly dynamic manner; this activation raises the question of whether *O*-GlcNAc can similarly serve as a rapidly inducible signal transduction mechanism.

This study was initiated to examine this question in detail and establish the relevance of *O*-GlcNAc in cellular responsiveness to external stimuli by using an established neutrophil model. Neutrophils are excellent tools for studying agonist-associated signaling and functional activities; they respond to a variety of agonists rapidly, the responses these agonists elicit are easilyassayed, and the signaling pathways through which these agonists operate are generally well defined. These attributes all certainly hold true for the chemoattractant agonist fMLF.

Focusing these studies on a single agonist, in this case fMLF, and on the effects of that agonist on a single system (cell type) makes it possible to follow in detail the effects

of *O*-GlcNAc from the level of protein modification and protein activity to the level of whole cell function. Thus, with respect to *O*-GlcNAc, if it is induced by fMLF, it should also be possible to determine how it is significant to these cells in terms of their functional responses as modulators of innate immunity. For example, do changes in protein *O*-GlcNAc have any impact on critical neutrophil functions elicited by fMLF, such as chemotaxis and respiratory burst activity? This is an important question because the physiological contexts in which this ubiquitous post-translational modification operates are still poorly defined. As mentioned above, *O*-GlcNAc was recently implicated in signaling stress responses under a variety of conditions (114). Inflammation and neutrophil activation are themselves stress responses (117). Therefore, it is plausible that *O*-GlcNAc plays an important functional role in the inflammatory responses of these cells, as it does for the stress response in other cells (114). In addition, it follows that, if *O*-GlcNAc modulates neutrophil function, it should do so through defined signaling pathways. This leads to a final question: what are the signal transduction pathways on which *O*-GlcNAcylated proteins work? As mentioned, these pathways are relatively well established in neutrophils, especially with respect to motility and respiratory burst activity (25), and as such should be readily accessible for dissection following treatments that alter protein *O*-GlcNAc.

In summary, these studies were developed to test the following overriding hypothesis that emerged from the above questions: *O*-GlcNAcylation is a highly dynamic signal in neutrophils that can be induced by fMLF to modulate the activities of signaling elements involved in the regulation of critical neutrophil functions such as respiratory burst activity and chemotaxis.

NEUTROPHILS EXHIBIT RAPID AGONIST-INDUCED INCREASES IN PROTEIN-ASSOCIATED *O*-GlcNAc

by

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Summary

A variety of cytoplasmic and nuclear proteins can be modified on serine and threonine residues by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc), although the effects of this modification on protein and cellular functions are not completely defined. The sugar donor for the *O*-GlcNAc transferase that catalyzes this post-translational modification is UDP-*N*-acetylglucosamine, a product of the hexosamine biosynthesis pathway. Here, the dynamics of the *O*-GlcNAc modification are examined in the physiological context of agonist-induced signal transduction using neutrophils. Formylated Met-Leu-Phe is shown to stimulate a rapid and transient increase in protein *O*-GlcNAcylation in both immunoblot and immunofluorescence imaging assays using *O*-GlcNAc-specific antibodies. In high performance liquid chromatography analyses of hexosamine biosynthesis pathway metabolic activity, shortterm exposure to an exogenous substrate of the hexosamine biosynthesis pathway, glucosamine, leads to increased glucosamine 6-phosphate and then UDP-*N*-acetylglucosamine levels. The glucosamine treatments also increase *O*-GlcNAcylation and augment the aforementioned formylated Met-Leu-Phe -associated increase. In functional assays, glucosamine pre-treatment selectively augments formylated Met-Leu-Phe -induced chemotaxis but has little effect on respiratory burst activity. Furthermore, augmenting levels of *O*-GlcNAc in the absence of agonist is sufficient to stimulate chemotaxis. These data demonstrate that neutrophils possess a functionally significant *O*-GlcNAcylation pathway that is robustly induced by stimulation with agonist. We propose that *O*-GlcNAcylation plays an important role in rapid and dynamic neutrophil signal transduction, especially with respect to chemotaxis.

Introduction

The addition of *N*-acetylglucosamine (GlcNAc) in β linkage to Ser/Thr residues (*O*-GlcNAc) is a dynamic post-translational modification that occurs on numerous cytoplasmic and nuclear proteins and is distinct from complex carbohydrates synthesized in the secretory pathway (1, 2). Proteins modified in this manner include nuclear pore components (2, 3), transcription factors (4, 5), cytoskeleton-associated proteins, and signaling enzymes (6, 7).

Early work on this modification suggested that it was dynamic and inducible and led to the prediction that *O*-GlcNAc would have a regulatory role analogous to phosphorylation (8-10). The cloning and characterization of enzymes responsible for the addition, UDP-GlcNAc: polypeptide *O*-β-*N*-acetylglucosaminyltransferase (OGT), and removal, neutral β-*N*-acetylglucosaminidase of *O*-GlcNAc moieties supported this idea (11-13). However, it has only been over the last few years that studies have begun to elucidate functional roles for *O*-GlcNAc and have uncovered mechanisms through which it may modulate cellular signaling pathways, and thereby cellular function, in health and disease. For example, altered *O*-GlcNAcylation has been extensively investigated with respect to its role in the pathogenesis of diabetes mellitus (6, 14-16) and may play a role in the etiology of certain cancers (17, 18) and Alzheimer disease (19, 20).

Many of the approaches used to investigate *O*-GlcNAc function have relied on the manipulation of hexosamine biosynthesis. Approximately 2-5% of the total glucose transported into cells feeds into the hexosamine biosynthesis pathway (HBP) to form glucosamine ($GlcNH_2$) 6-phosphate ($GlcNH_2$ 6-P) and ultimately UDP-*N*-acetylglucosamine (UDP-GlcNAc), the sugar donor for OGT (21). OGT activity has been found to be sensi-

tive to relatively small changes in substrate availability over a wide range of substrate concentrations and, additionally, recognizes different acceptor proteins at different concentrations of UDP-GlcNAc (22). Thus, as several studies have shown, when cells in culture (4, 14, 23) or tissues *in vivo* (15) are exposed to hyperglycemic conditions they exhibit enhanced levels of *O*-GlcNAc that can be blocked by HBP inhibitors. This indicates that elevated levels of glucose lead to enhanced HBP flux and UDP-GlcNAc formation and thereby enhanced *O*-GlcNAc. Exogenous $GlcNH_2$, which is not normally present in the cellular environment at appreciable levels, is also transported into cells and preferentially metabolized through this pathway, resulting in enhanced *O*-GlcNAc (6, 21, 23).

Proponents of *O*-GlcNAc as an important protein modification theorize that *O*-GlcNAc regulates critical aspects of protein biology, including protein stability, subcellular localization, and protein-protein interactions. The current body of research in this area supports these ideas, and several reviews have recently emerged expounding their various facets (1, 7, 24). However, important questions remain. Foremost among these is whether *O*-GlcNAc can serve as a rapid, highly inducible signal transduction mechanism akin to phosphorylation, the archetypal regulatory mechanism for the coupling of extracellular signals to specific cell responses.

The bulk of the work on the functional significance of *O*-GlcNAc has relied on longterm treatments, hours or days, with either high glucose (4, 14, 15) or $GlcNH_2$ (6, 3), or the use of pharmacological agents (6, 25-27) that inhibit specific enzymes in the pathways affecting *O*-GlcNAcylation to manipulate this protein modification. Lymphocytes provided early evidence that *O*-GlcNAcylation can be a dynamic process, because pharmacological mitogens were shown to induce changes in cellular *O*-GlcNAc over times as

short as 1 h (9). In addition, certain pharmacological agents that bypass physiologically relevant agonist- and receptor-specific signaling mechanisms induce relatively rapid changes in *O*-GlcNAc. In particular, pharmacological treatments with the calcium ionophore A23187 and the protein phosphatase inhibitor okadaic acid have been shown to induce changes in *O*-GlcNAc within one minute, suggesting that *O*-GlcNAc responses may indeed proceed quite rapidly (28). These examples are often used as corroborative evidence of the *O*-GlcNAc potential as a signaling mechanism that is analogous to phosphorylation. There is, however, only limited evidence of physiological agonist-induced, receptor-mediated changes in *O*-GlcNAcylation. Specifically, insulin infusions over several hours lead to increases in *O*-GlcNAc, although it is likely that these responses are due to an increase in glucose transport and a slowly progressive increase in HBP flux rather than a direct effect of insulin on the *O*-GlcNAcylation machinery (6, 29, 30).

Neutrophils (polymorphonuclear leukocytes or PMNs) respond to a large and diverse group of stimuli over a range of times, resulting in a variety of metabolic and functional responses. These responses, which include degranulation, phagocytosis, chemotaxis, a respiratory burst, and alterations in gene expression, ultimately lead to microbe killing. Within this context PMNs have served as useful models for studying the signal transduction mechanisms involved in cellular stimulation. One of the most commonly used PMN stimuli, the chemotactic tripeptide formylated-methionine-leucine-phenylalanine (fMLF), binds to cell surface receptors and induces protein phosphorylation within tens of seconds (31-33). These phosphorylation events are central to a diverse set of signaling pathways, leading, for example, to chemotaxis and the production of reactive oxygen species (34-38).

This study was initiated to examine the relevance of *O*-GlcNAc in cellular responsiveness to external stimuli using an established PMN model. We report here that PMNs possess a functionally significant HBP and associated *O*-GlcNAcylation mechanism. In doing so, we also provide evidence of rapid and robust agonist-induced changes in *O*-GlcNAcylation. We show that PMNs respond rapidly to both $GlcNH_2$ and agonist, leading to similar and additive increases in protein *O*-GlcNAcylation. Our data support the premise that protein *O*-GlcNAcylation is a highly dynamic signaling mechanism capable of rapidly transducing receptor-associated signals to influence cellular function.

Experimental Procedures

PMN Isolation

Whole blood from volunteers was obtained by venipuncture and layered onto a double discontinuous gradient formed with equal volumes of Histopaque-1077 (Sigma) over Histopaque-1119 (Sigma) (39). The blood was centrifuged at 700 x *g* for 30 min. Granulocytes were collected from the 1077/1119 interface and washed in Hank's buffered salt solution buffered with 10 mM HEPES, pH 7.4 . Contaminating red blood cells were lysed by incubation in 0.15 M NH_4Cl/1 mM $KHCO_3$/0.1 mM EDTA for 5 min at 37 °C. The granulocytes were then washed twice in Hank's buffered salt solution and resuspended in Hank's buffered salt solution. The resulting samples were >95% PMNs as indicated by the polymorphic nature of their nuclei (see "Immunofluorescence Microscopy" below). Cell viability was assessed through Trypan Blue (Sigma) exclusion.

Immunoblotting

2 x 10^6 PMNs were treated as indicated, directly lysed in 5X sample buffer (0.3 M Tris·HCl/5% SDS/50% glycerol/0.025% bromophenol blue/5% mercaptoethanol) and boiled for 5 min. 7.5 x 10^5 cell equivalents per lane (~60 μg of protein) were separated by SDS-PAGE (40) and transferred to Immobilon-P (Millipore). Immunoblotting was performed using a rapid immunodetection method for Immobilon-P (Millipore Tech Note TN051). Briefly, the membranes were equilibrated in methanol and air dried. The dry membrane was incubated with a 1:1000 dilution of anti-*O*-GlcNAc antibody CTD110.6 (41) (Covance) in 1% casein/phosphate-buffered saline (PBS) (Pierce) with 0.01% Tween 20 for 2 h and then washed three times in PBS. To demonstrate *O*-GlcNAc-specific immunoreactivity, 10 mM GlcNAc was added during the primary antibody incubation (41). The membrane was then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM (Calbiochem) in 1% casein/PBS with 0.01% Tween 20 for 1 h. After further washing in PBS the immunoblots were developed with enhanced chemiluminescence (SuperSignal West Pico; Pierce).

Immunofluorescence Microscopy

PMNs at 1.25 x 10^6 cells/ml were treated as indicated. The cells were fixed by adding an equal volume of 6% formaldehyde (Tousimis)/PBS for 30 min and resuspended in PBS. The cells were cytospun onto glass coverslips at 500 x *g* for 5 min. The coverslips were post-fixed in 3% formaldehyde/PBS for 10 min and washed with PBS. The PMNs were permeablized with methanol (-20 °C) for 2 min. After washing, the coverslips were blocked for 30 min at room temperature in 1% casein/PBS with 0.2% Tween

20 and then incubated with a 1:250 dilution of anti-*O*-GlcNAc antibody CTD110.6 in 1% casein/PBS with 0.2% Tween 20 for 60 min at 37 °C. To demonstrate *O*-GlcNAc-specific immunoreactivity, 100 mM GlcNAc was added during the primary antibody incubation. After washing, the coverslips were then blocked for 10 min at room temperature with 10% normal goat serum (Sigma)/PBS with 0.2% Tween 20 and then incubated with a 1:250 dilution of Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes) in 10% normal goat serum/PBS with 0.2% Tween 20 for 45 min at room temperature. The coverslips were finally washed and mounted with 9:1 glycerol/PBS. Hoechst 33258 (Molecular Probes), 1:1000 dilution of 10 mg/ml stock, was used when indicated to counter-stain nuclei (>95% of cells stained had polymorphic nuclei consistent with PMN nuclear morphology). Quantification of fMLF-induced *O*-GlcNAc label by fluorescence intensity was performed using Image J.

$GlcNH_2$ 6-P and UDP-GlcNAc Determinations

5 x 10^6 PMNs were treated as indicated and analyzed for $GlcNH_2$ 6-P and UDP-GlcNAc as previously described (42). Briefly, the cells were centrifuged, and the pellet extracted with 100 µl of 0.3 M perchloric acid. The extract was centrifuged for 10 min at 4 °C at 14,000 x *g*, and 200 µl of 1:4 trioctylamine:1,1,2-trichlorotrifluoroethane (freon) was mixed with the resulting supernatant. The mixture was centrifuged for 5 min at 4 °C at 14,000 x *g*, and the aqueous phase (top) was stored at -80 °C for up to 1 week. For high performance liquid chromatography determination of UDP-GlcNAc the aqueous phase was run on a strong anion exchange column (Partisil SAX; Thermo) with the detector set to 262 nm. The flow rate was 2 ml/min, with the mobile phases consisting of 5 mM

$NH_4H_2PO_4$, pH 2.8, and 750 mM $NH_4H_2PO_4$, pH 3.7, for buffers A and B, respectively, with a linear gradient from 0% to 100% buffer B over 40 min. The retention time for UDP-GlcNAc was ~17 min. For the high performance liquid chromatography determination of $GlcNH_2$ 6-P 100 µl of the aqueous phase was mixed with 200 µl of 6 mM O-pthalaldehyde (Sigma)/1% ethanol/0.2% mercaptoethanol/0.2 M boric acid, pH 9.7, for 1 min followed by neutralization with 400 µl of 100 mM NaH_2PO_4. The sample was then loaded onto a C-18 column (Ultrasphere; Beckman Coulter) with the detector set to 340 nm. The flow rate was 0.5 ml/min, with the mobile phases consisting of 90% 16.7 mM NaH_2PO_4, pH 7.2/5% iso-propanol/5% acetonitrile and 76% 19.7 mM NaH_2PO_4, pH 7.2/12% iso-propanol/12% acetonitrile for buffers A and B, respectively, with a linear gradient from 0% to 100% B over 15 min followed by a linear gradient of 100% to 0% buffer B over 10 min. The retention time for $GlcNH_2$ 6-P was ~9.5 min. The data were analyzed and quantified as area under the curve by System Gold Nouveau software (Beckman Coulter). For determination of nanomoles UDP-GlcNAc/mg of protein and picomoles $GlcNH_2$ 6-P/mg protein, UDP-GlcNAc (Sigma) and $GlcNH_2$ 6-P (Sigma) standards were run to establish molar reference values. PMN cell equivalents lysed in 1% Nonidet P-40/0.5% sodium deoxycholate/50 mM Tris-HCl, pH 7.4/150 mM NaCl were assayed using the Lowry method (43) for protein concentration determination.

Chemotaxis Assays

2×10^5 PMNs were pre-treated as indicated, washed, resuspended in Dulbecco's modification of Eagle's medium/5% bovine serum albumin and then placed in the upper filter plate of a MultiScreen-MIC chemotaxis plate (Millipore) with 3-µm membrane

pores. The lower receiver plate contained Dulbecco's modification of Eagle's medium and 100 μM fMLF where indicated. Basal chemotaxis was assessed in the absence of fMLF. The chemotaxis chambers were placed in a 37 °C incubator for 45 min. The filter plate was then carefully removed, and migrating cells were counted by microscopic examination of receiver plates (44).

Respiratory Burst Assays

PMN respiratory burst activity was assessed by luminol-dependent chemiluminescence (45). The PMNs were pre-treated where indicated, and all samples were rapidly washed before being assayed (the presence of free $GlcNH_2$ in the chemiluminescence buffer was found to inhibit chemiluminescence readings). Samples were prepared by mixing 10^6 washed PMNs into Hank's buffered salt solution/10 μM luminol/100 μM sodium azide/10 Units/ml horseradish peroxidase. Chemiluminescence was monitored at room temperature in a tube-based luminometer (Optocomp 1; MGM Instruments) without stirring or shaking. Baseline readings were established over 30 sec followed by stimulation with fMLF as indicated.

Results

PMNs Display Rapid Agonist-induced Changes in O-*GlcNAc*

The formylated peptide fMLF is a well established and widely used PMN agonist. It rapidly activates a diverse group of signal transduction pathways by engaging a specific G-protein-coupled receptor (33). We used fMLF to assess whether receptor-associated stimulation could signal protein *O*-GlcNAcylation. 2 min of stimulation with

100 nM fMLF led to increased protein-associated *O*-GlcNAc as determined by immunoblot analyses of whole cell extracts with the *O*-GlcNAc-specific antibody CTD110.6 (Fig. 1*A*). A variety of proteins with molecular weights above ~50 kDa were observed, with some variation in the banding pattern and/or intensity of some bands from experiment to experiment, as might be expected for primary PMN isolates. Additional analyses revealed that *O*-GlcNAcylation begins within 1 min of stimulation and progresses through 5 min (Fig. 1*B*). In each experiment the specificity of CTD110.6 immunoreactivity was established by competitively blocking antibody binding with free GlcNAc.

Although the *O*-GlcNAc-specific antibody CTD110.6 has been successfully used previously in immunoblot assays (41), it has not been evaluated with respect to immunocytochemical microscopy. We developed a protocol using CTD110.6 in a fluorescence-based immunocytochemical assay as an alternate and complementary method of examining changes in *O*-GlcNAc modifications. 100 nM fMLF increased *O*-GlcNAc over 2 min of stimulation (Fig. 2*A*). We obtained low background, antibody-specific signal using formaldehyde fixation followed by methanol permeation. The permeation agent was a critical variable in developing this assay, as commonly used agents such as Triton and acetone yielded high nonspecific backgrounds (data not shown). The agonist-induced CTD110.6 signal was specific for protein-associated *O*-GlcNAc, as it was competitively blocked by free GlcNAc. These results paralleled our immunoblot data.

CTD110.6 labeled both the nucleus and cytoplasm in resting and stimulated cells. The cells displayed two predominant patterns of label: a less intense, diffuse, and relatively homogenous label and a comparatively intense, non-homogenous, punctate label

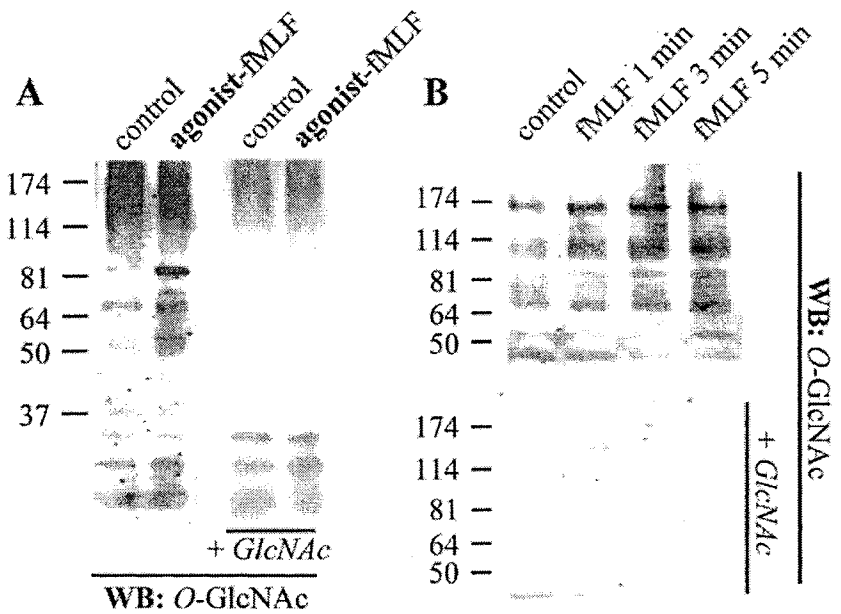


FIG. 1. **Polymorphonuclear leukocytes display rapid agonist-induced protein *O*-linked β-*N*-acetylglucosamine as assessed by -specific immunoblots.** Immunoblots were performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6. The specificity of the antibody for protein *O*-linked β-*N*-acetylglucosamine (O-*GlcNAc*) was determined by adding 10 mM *N*-acetylglucosamine to the primary antibody dilution buffer (indicated as +*GlcNAc*). *A*, polymorphonuclear leukocytes were left unstimulated (*control*) or stimulated with agonist, 100 nM formylated methionine-leucine-phenylalanine (*fMLF*), for 2 min. *WB*, Western blot. *B*, polymorphonuclear leukocytes were left unstimulated (*control*) or stimulated with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) for 1, 3, or 5 min. *WB*, Western blot.

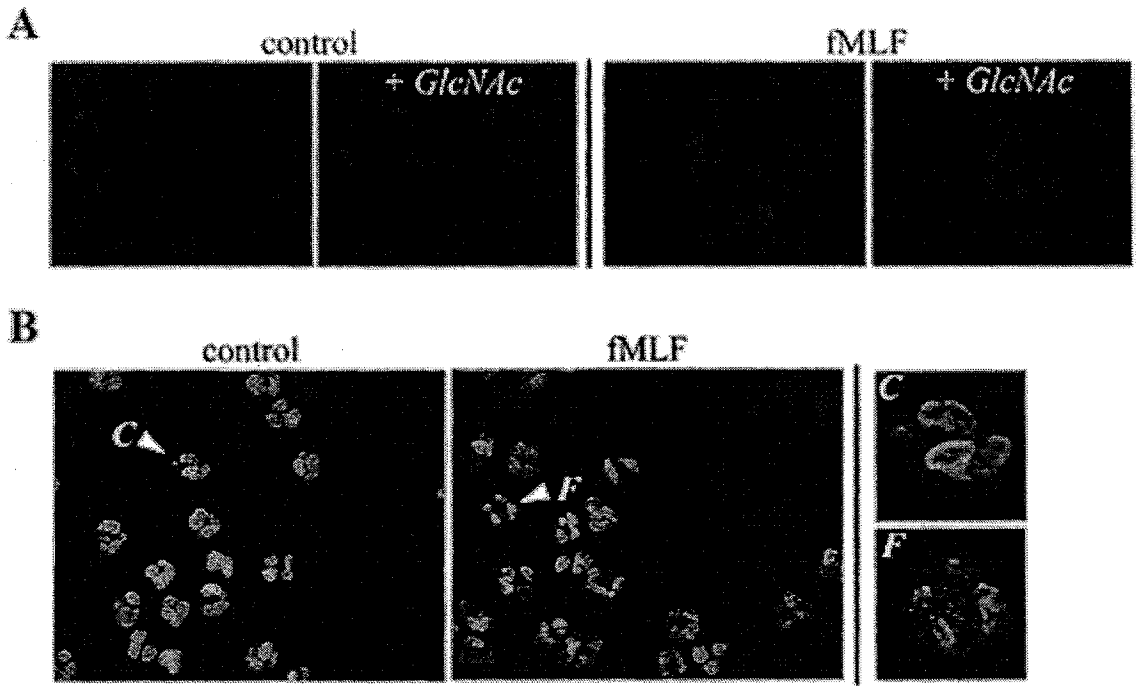


FIG. 2. **Immunofluorescence microscopy reveals rapid agonist-induced protein *O*-linked β-*N*-acetylglucosamine.** Immunofluorescence labeling was performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6. The specificity of this antibody for protein *O*-linked β-*N*-acetylglucosamine was assessed by adding 100 mM *N*-acetylglucosamine to the primary antibody dilution buffer (indicated as *+GlcNAc*). *A*, polymorphonuclear leukocytes were left unstimulated (*control*) or stimulated with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) for 2 min. Two predominant labeling patterns were evident: a relatively intense punctate label and a less intense, diffuse and homogenous label. *B*, the more intense, punctate *O*-linked β-*N*-acetylglucosamine label was limited to the cytoplasm, as was evident when the cells were counterstained using the nuclear stain Hoechst 33258. Polymorphonuclear leukocytes were left unstimulated (*control*) or stimulated with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) for 2 min. Higher magnification images of selected cells revealed a punctate label that was localized exclusively to the cytoplasm (the *arrows* in the low magnification images that are labeled as *C* and *F*, *control*, and *fMLF*, respectively, are the individual cells shown in the higher magnification views).

that, in contrast to a Hoechst counterstain that clearly defined the polymorphic multi-lobed PMN nucleus, was restricted to the cytoplasm. The punctate cytoplasmic label displayed the major increase in fluorescence intensity upon agonist stimulation (Fig. 2*B*). Labeling of the nucleus was more diffuse and considerably less intense than the punctate cytoplasmic label, although it too increased with stimulation. This was somewhat unexpected, because a variety of *O*-GlcNAcylated proteins are known to be nuclear, and a more intense nuclear signal might be anticipated (7). It is not clear whether this was a property of the particular labeling protocol used, a feature of *O*-GlcNAc-modified proteins in PMNs, or a reflection of a specific property of the antibody and/or the cell.

Of further interest, this assay also proved to be a sensitive means of assessing the temporal dynamics of agonist-induced *O*-GlcNAcylation. Increases in protein *O*-GlcNAcylation were evident within 30 s of stimulation with fMLF and returned to near resting levels after 10 min (Fig. 3). This provided further support for the notion that *O*-GlcNAc is a rapidly inducible post-translation protein modification.

PMNs Possess a Functional HBP That Can Use Exogenous $GlcNH_2$ to Form the Metabolic Precursors for Protein O-*GlcNAcylation*

The HBP plays an integral role in *O*-GlcNAc signaling, providing UDP-GlcNAc, the necessary substrate to drive OGT activity and *O*-GlcNAcylation (21). Having established that PMNs posses a robust and rapidly inducible *O*-GlcNAcylation mechanism, we next asked what the effects of increasing flux through the HBP would have in PMNs. $GlcNH_2$ is an exogenous substrate for the HBP that is commonly used to drive HBP-dependent *O*-GlcNAcylation (6, 21, 23). On entering a cell it is phosphorylated by hexokinase to form $GlcNH_2$ 6-P, thus directly entering the HBP. Further processing

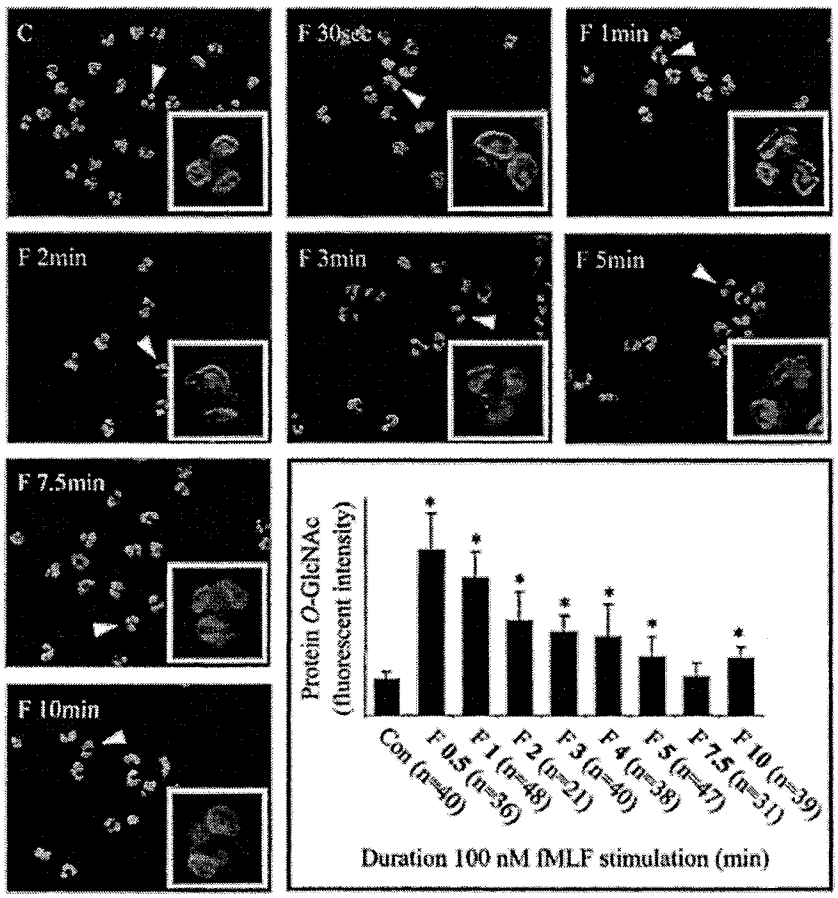


FIG. 3. **Immunofluorescence microscopy is an effective means of determining the temporal dynamics of agonist-induced protein *O*-linked β-*N*-acetylglucosamine.** Immunofluorescence labeling was performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6, and Hoechst 33258 was used as a nuclear counterstain. formylated methionine-leucine-phenylalanine (*fMLF*) induced robust cytoplasmic *O*-linked β-*N*-acetylglucosamine (O-*GlcNAc*) within 30 s that continued for at least 5 min, after which it appeared to decrease toward resting levels. Polymorphonuclear leukocytes were left unstimulated (*C*) or stimulated with 100 nM formylated methionine-leucine-phenylalanine for various times ranging from 30 s (*F 30s*) to 10 min (*F 10min*). The *inset* shows quantification of formylated methionine-leucine-phenylalanine-induced *O*-linked β-*N*-acetylglucosamine label by fluorescence intensity (n = number of cells examined for each condition; *, indicates $p < 0.05$ in comparison to control as evaluated by Student's t-test).

yields UDP-GlcNAc, the HBP product necessary for *O*-GlcNAcylation.

We examined $GlcNH_2$ 6-P and UDP-GlcNAc levels in $GlcNH_2$-treated PMNs by high performance liquid chromatography. Resting $GlcNH_2$ 6-P levels were about 20-fold less than UDP-GlcNAc levels, ~0.06 pmol/mg of protein *versus* ~1.4 nmol/mg of protein (Fig. 4). UDP-GlcNAc was the predominant sugar nucleotide in PMNs and was a major phospho-nucleotide, along with ADP, ATP, and UDP-glucose (data not shown). This suggests that a relatively abundant pool of UDP-GlcNAc is important for normal PMN physiology and function. Short duration treatments with 10 mM $GlcNH_2$ led to the production of both $GlcNH_2$ 6-P and UDP-GlcNAc. $GlcNH_2$ 6-P levels rose following $GlcNH_2$ treatment, doubling over 2 min and increasing ~8-fold by 60 min (Fig. 4*A*), indicating that $GlcNH_2$ rapidly entered the cells and was quickly phosphorylated by hexokinase. The increase in UDP-GlcNAc levels clearly followed the rise in $GlcNH_2$ 6-P (Fig. 4*B*), as might be expected for HBP processing. Although the percent increase in UDP-GlcNAc was significantly smaller than that for $GlcNH_2$ 6-P at any given time (for example, 60% *versus* 700% after 60 min), the molar increase in UDP-GlcNAc was greater than that for $GlcNH_2$ 6-P (an increases of 0.83 *versus* 0.42 nmol/mg of protein after 60 min). No drop in cellular ATP levels was observed for $GlcNH_2$ treatment (data not shown). In fMLF-treated cells there was no apparent change in $GlcNH_2$ 6-P or UDP-GlcNAc levels with stimulation (data not shown).

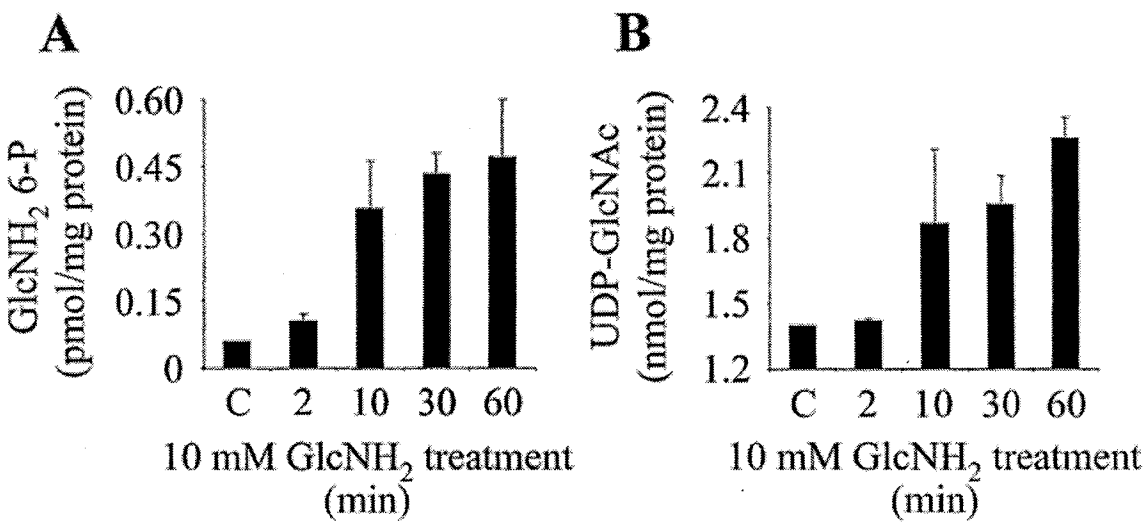


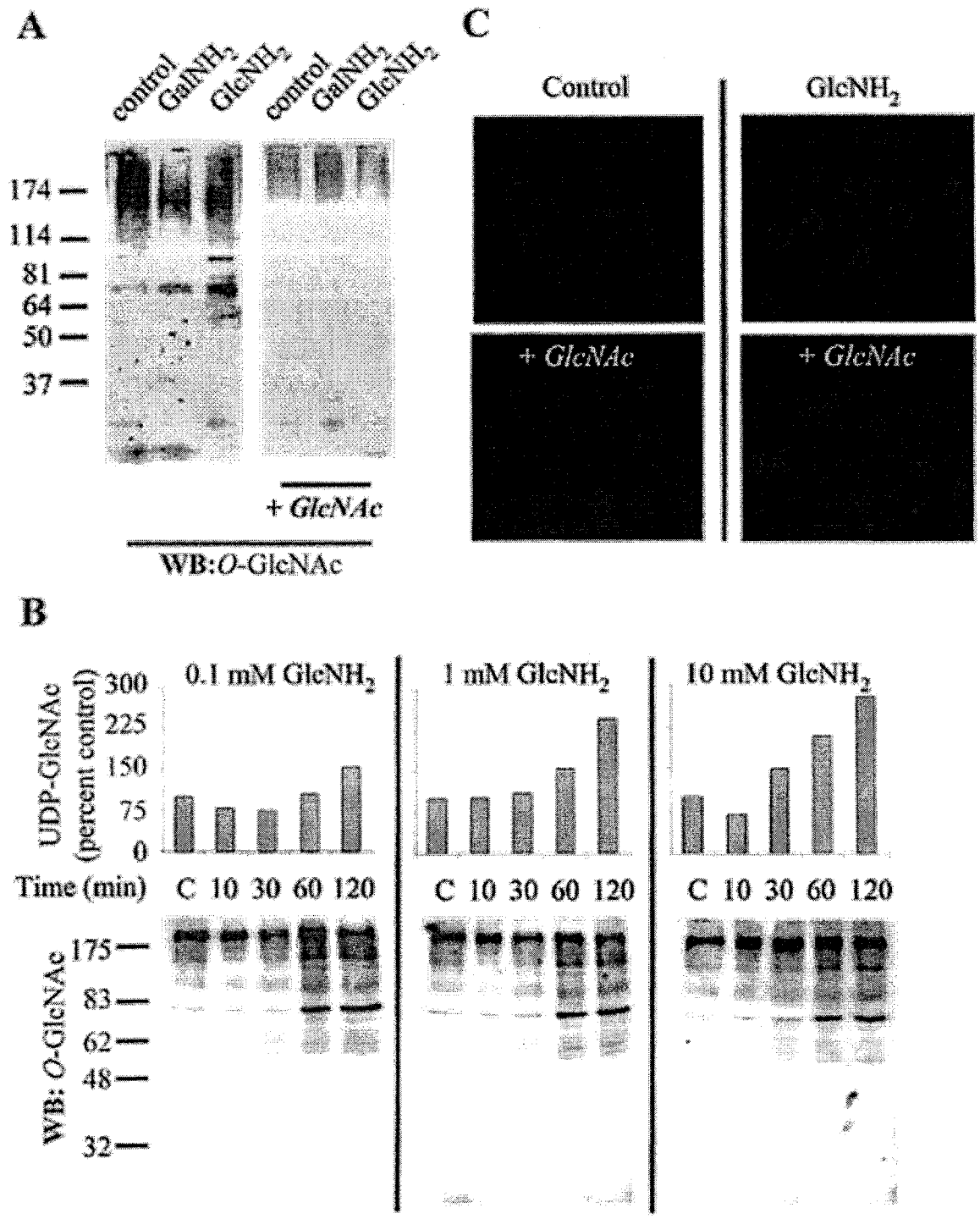
FIG. 4. **Polymorphonuclear leukocytes metabolize exogenous glucosamine into metabolic precursors that are used in the pathway leading to protein *O*-linked β-*N*-acetylglucosamine.** *A*, glucosamine 6-phosphate (*$GlcNH_2$ 6-P*) levels in polymorphonuclear leukocytes treated with glucosamine (*$GlcNH_2$*). Polymorphonuclear leukocytes were left untreated (*C*) or treated with 10 mM glucosamine for 2, 10, 30 or 60 min at 37 °C. *O*-pthalaldehyde-conjugated acid extracts ($n = 3$ for each condition) were analyzed on a carbon-18 column using high performance liquid chromatography. *B*, uridine diphosphate-*N*-acetylglucosamine (*UDP-GlcNAc*) levels in polymorphonuclear leukocytes treated with glucosamine (*$GlcNH_2$*). Polymorphonuclear leukocytes were left untreated (*C*) or treated with 10 mM $GlcNH_2$ for 2, 10, 30, or 60 min at 37 °C. Acid extracts ($n = 3$ for each condition) were analyzed on a strong anion exchange column using high performance liquid chromatography.

$GlcNH_2$ Increases Protein-associated O-*GlcNAc under the Same Conditions That Increase UDP-GlcNAc*

We next determined if the observed $GlcNH_2$-induced changes in HBP flux were associated with alterations in *O*-GlcNAc. Short duration $GlcNH_2$ treatment, 10 mM for 30 min, enhanced protein *O*-GlcNAc in whole cell extracts as determined by immunoblotting with the *O*-GlcNAc-specific antibody CTD110.6 (Fig. 5*A*). A group of proteins bearing *O*-GlcNAc with a wide range of molecular weights above ~50 kDa was observed, with some variation in the banding pattern from experiment to experiment. The overall banding pattern was similar to that induced by fMLF (refer to Fig. 1, *A* and *B*). The specificity of these immunoblots in identifying proteins modified through $GlcNH_2$-specific metabolism was confirmed by pre-treatment with 10 mM galactosamine, a stereoisomer of $GlcNH_2$, that did not lead to increased *O*-GlcNAc in the same time frame. The specificity of CTD110.6 for detecting *O*-GlcNAcylated proteins was again established through competitive binding assays using free GlcNAc.

To investigate the relationship between increased UDP-GlcNAc and protein *O*-GlcNAc in our system, aliquots from the same PMN preparation were taken for each experimental condition (Fig. 5*B*). These experiments used PMNs from a single donor and cell isolation, eliminating day-to-day and inter-donor variation. We found that significant changes in $GlcNH_2$-induced protein *O*-GlcNAcylation corresponded with only modest increases in UDP-GlcNAc. UDP-GlcNAc levels and *O*-GlcNAcylation were dependent on the $GlcNH_2$ dose and pre-treatment duration: higher doses and longer pre-treatments enhanced UDP-GlcNAc and *O*-GlcNAc, whereas lower doses and shorter pre-treatments were either without effect or produced more modest increases. There was an apparent threshold for robust *O*-GlcNAcylation at 60 min of treatment for all concentrations

FIG. 5. **Glucosamine increases protein *O*-linked β-*N*-acetylglucosamine under the same conditions that increase uridine diphosphate-*N*-acetylglucosamine.** *A*, immunoblots were performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6. The specificity of the antibody for protein *O*-linked β-*N*-acetylglucosamine (O-*GlcNAc*) was assessed by adding 10 mM *N*-acetylglucosamine to the primary antibody dilution buffer (indicated as +*GlcNAc*). Substrate driven *O*-linked β-*N*-acetylglucosamine was assessed by adding 10 mM *N*-acetylglucosamine ($GlcNH_2$) or 10 mM *N*-acetylgalactosamine ($GalNH_2$) for 30 min at 37 °C. Control polymorphonuclear leukocytes were left untreated. *WB*, Western blot. *B*, single matched uridine diphosphate-*N*-acetylglucosamine (*UDP-GlcNAc*) and *O*-linked β-*N*-acetylglucosamine (O-*GlcNAc*) (immunoblots with CTD110.6) analyses for polymorphonuclear leukocytes treated with 0.1, 1, or 10 mM *N*-acetylglucosamine ($GlcNH_2$) for 10, 30, 60 and 120 min each at 37 °C. Control cells were left untreated. *C*, immunofluorescence labeling was performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6. The specificity of this antibody for protein *O*-linked β-*N*-acetylglucosamine was assessed by adding 100 mM *N*-acetylglucosamine ($GlcNH_2$) to the primary antibody dilution buffer (indicated as +*GlcNAc*). Polymorphonuclear leukocytes remained untreated (*left*) or were treated with 10 mM *N*-acetylglucosamine for 30 min at 37 °C (*right*). *WB*, Western blot.



tested, although this did not clearly reflect a defined level of UDP-GlcNAc. However, there was a trend for progressively elevated UDP-GlcNAc to correspond to progressively enhanced *O*-GlcNAc (for the representative experiment shown in Fig. 5*B* this was especially evident for the 10 and 1 mM $GlcNH_2$ conditions).

We used our previously described fluorescence-based immunocytochemical assay again as an alternate and complementary method of evaluating $GlcNH_2$-induced *O*-GlcNAcylation. Cells treated with 10 mM $GlcNH_2$ for 30 min showed a significantly increased fluorescent signal *versus* untreated controls (Fig. 5*C*). The signal was specific for *O*-GlcNAc, because it was competitively blocked by free GlcNAc. These results paralleled and confirmed our immunoblot data. Interestingly, the $GlcNH_2$-associated increase in CTD110.6 label was, as it was by immunoblot, grossly similar to that observed for fMLF (refer to Fig. 2, *A* and *B*). The effects of $GlcNH_2$ were most readily apparent in the cytoplasm, with a relatively large increase in the punctate label. The more diffuse and homogenous labeling pattern, present in the cytosol and the nucleus, was also increased by $GlcNH_2$ treatment.

$GlcNH_2$ Treatment Amplifies fMLF-induced Protein O-*GlcNAcylation*

We next asked if $GlcNH_2$ was effective in conjunction with fMLF as a means of manipulating agonist-associated *O*-GlcNAcylation. Combined $GlcNH_2$ and agonist treatments were additive for *O*-GlcNAcylation, with short duration $GlcNH_2$ pretreatments, which do not significantly increase *O*-GlcNAc by themselves (refer to Fig. 5*B*), leading to noticeably increased fMLF-induced *O*-GlcNAc (Fig. 6). These additive effects were most clearly evident at higher $GlcNH_2$ concentrations. These experiments

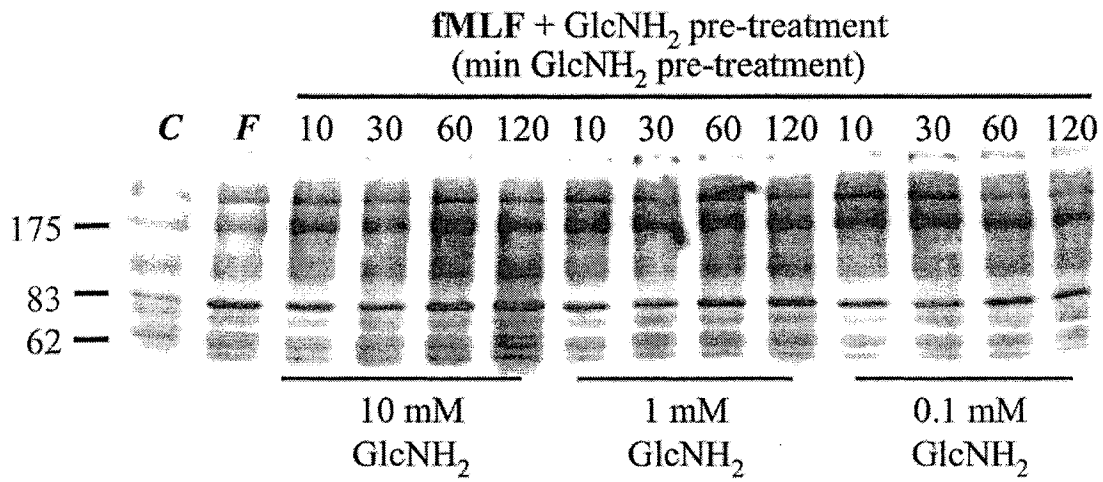


FIG. 6. **Glucosamine treatment modifies formylated methionine-leucine-phenylalanine-induced protein *O*-linked β-*N*-acetylglucosamine.** Immunoblots were performed using the anti-*O*-linked β-*N*-acetylglucosamine antibody CTD110.6. Polymorphonuclear leukocytes were pretreated as indicated with 10, 1, or 0.1 mM glucosamine (*$GlcNH_2$*) for 10, 30, 60, and 120 min at 37 °C. The cells were then stimulated with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) for 2 min (indicated by *F* for the non-pretreated sample). The control sample, *C*, was not stimulated and was not pretreated.

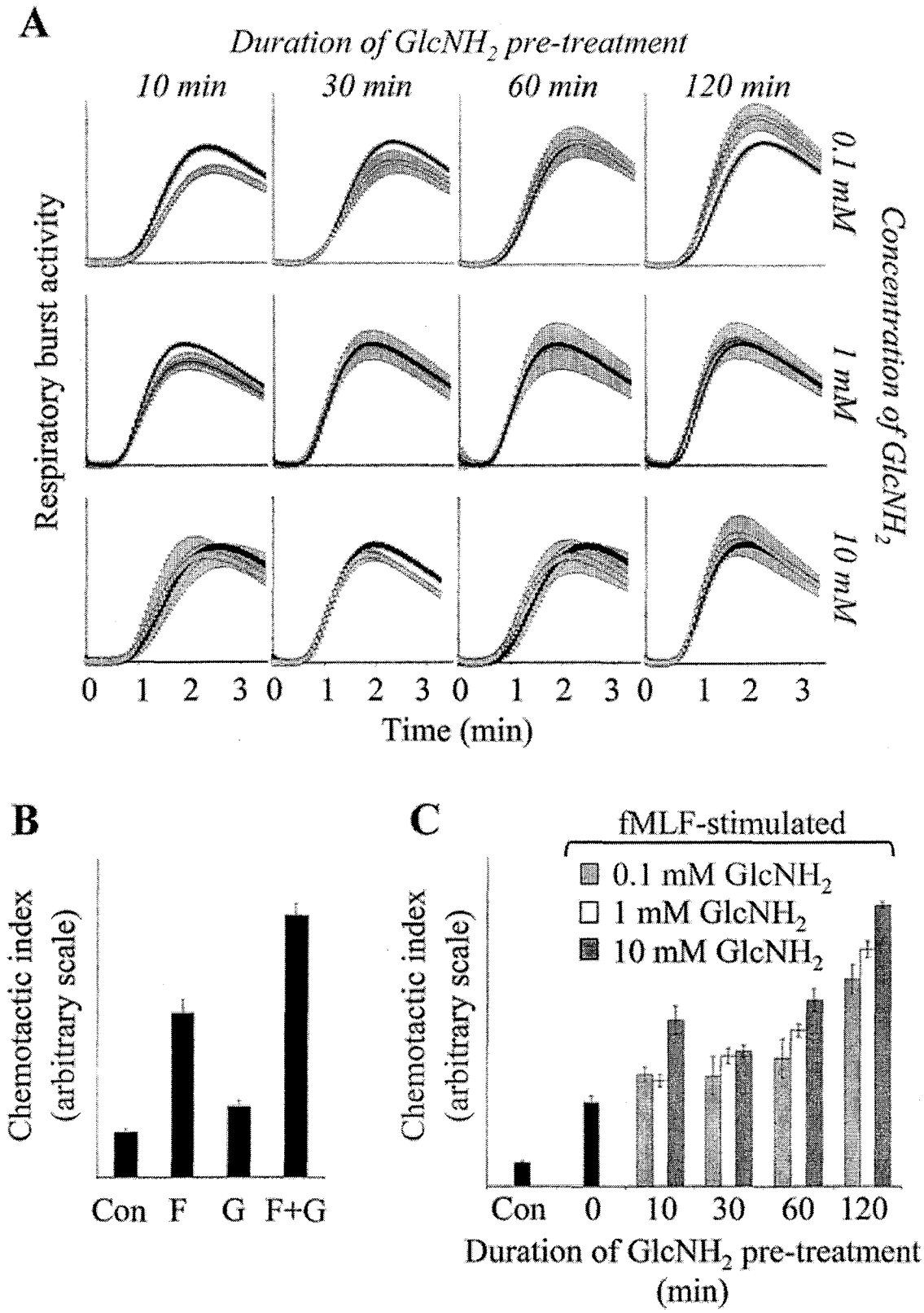
also confirmed that the pattern of *O*-GlcNAcylation due to fMLF approximated that observed for $GlcNH_2$.

$GlcNH_2$ Selectively Modulates PMN Functional Outputs

Having shown that $GlcNH_2$ pre-treatment can be used to modulate fMLF-induced *O*-GlcNAcylation, we attempted to elucidate what effect(s) this had on fMLF-associated function. This would provide an initial determination of whether changes in *O*-GlcNAc have a signaling role. We examined the effect of $GlcNH_2$ treatment on agonist-induced respiratory burst (defined by the production of reactive oxygen species) and chemotactic activities. $GlcNH_2$ pre-treatments of various durations, 10-120 min, and concentrations, 0.1-10 mM $GlcNH_2$, had little effect (in all cases statistically non-significant, $p > 0.05$) on the respiratory burst elicited by 100 nM fMLF in luminol-dependent assays (Fig. 7*A*). There was a trend toward reduced and slower onset activity for short duration $GlcNH_2$ pre-treatments, 10 and 30 min, and somewhat enhanced and faster onset activity for long duration pre-treatments, 120 min. The $GlcNH_2$ treatments did not affect cell viability or morphology (data not shown).

An initial assessment of the effect of short duration $GlcNH_2$ pre-treatment, 10 mM for 30 min, on chemotaxis revealed that $GlcNH_2$ significantly increases both basal and fMLF-induced activity (60 and 63%, respectively) in simple 45-min Boyden chamber-type assays (Fig. 7*B*). These data supported the possibility that $GlcNH_2$ selectively modulates only certain aspects of neutrophil physiology. We further defined $GlcNH_2$-enhanced chemotaxis by altering both the duration of $GlcNH_2$ pre-treatment and the concentration of $GlcNH_2$ used (Fig. 7*C*). The chemotactic response was dependent on the

FIG. 7. **Glucosamine modulates polymorphonuclear leukocyte chemotaxis but not respiratory burst activity.** *A*, respiratory burst activity was assessed by luminol-dependent chemiluminescence. Polymorphonuclear leukocytes ($n = 3$ for all conditions) were pretreated with 0.1, 1, or 10 mM glucosamine ($GlcNH_2$) for 10, 30, 60, and 120 min at 37 °C and then stimulated with 100 nM formylated methionine-leucine-phenylalanine (*open circles* ○). Control samples were not pretreated with glucosamine, but were stimulated with 100 nM formylated methionine-leucine-phenylalanine (*closed circles* ●). For all cases formylated methionine-leucine-phenylalanine stimulation was preceded by 30 s of baseline recording. *B*, chemotactic activity was assessed in Boyden chamber-type migration assays ($n = 3$ for all conditions). Polymorphonuclear leukocytes did not receive a pretreatment, *Con* and *F*, or were pretreated with 10 mM glucosamine 30 min at 37 °C, *G* and *F+G*. Basal levels of chemotaxis were monitored in the absence of chemotactic factor, *Con* and *G*, and fMLF-induced chemotaxis was monitored in the presence of 100 nM fMLF, *F* and *F+G*. Assays were carried out at 37 °C for 45 min. *C*, chemotactic activity was assessed in 96-well Boyden chamber-type migration assays ($n = 3$ for all conditions). Polymorphonuclear leukocytes were not pretreated, *Con and 0,* or pretreated with 0.1 mM (*light grey bars* ▩), 1 mM (*white bars* □) or 10 mM (*dark grey bars* ■) glucosamine ($GlcNH_2$) for 10, 30, 60 and 120 min at 37 °C as indicated. 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) was used to initiate chemotaxis in samples labeled *0*, *10*, *30*, *60*, and *120* min. The control sample, *Con*, was not stimulated with chemotactic factor (basal chemotaxis). Assays were carried out at 37 °C for 45 min.



$GlcNH_2$ dose and pre-treatment duration: higher doses and longer pre-treatments clearly enhanced chemotactic activity toward fMLF, whereas lower doses and shorter pre-treatments were without significant effect. Significantly enhanced chemotaxis was observed for all $GlcNH_2$ doses tested by 120 min of pre-treatment, with 10 mM $GlcNH_2$ requiring as little as 10 min of pre-treatment and 1 mM requiring ~60 min.

Discussion

Protein *O*-GlcNAcylation is emerging as an important post-translational modification that regulates protein function. However, the physiological contexts in which rapid changes in the level of *O*-GlcNAc occur are only beginning to be elucidated. Changes in *O*-GlcNAcylation are implicated in the pathogenesis of diabetes mellitus, a disease characterized by chronic hyperglycemia (6, 14, 15), and have also been suggested to serve as a nutrient sensor, in which *O*-GlcNAc signals are modulated through nutrient availability (46, 47). Recently, work by Zhang has suggested that *O*-GlcNAc may couple proteasomes to the general metabolic state of the cell (48), and Zachara has provided evidence that *O*-GlcNAc modulates stress-activated signaling networks and may be integral to cellular survival responses (49). In addition, *O*-GlcNAcylation is also often compared with phosphorylation (8-10), which is fundamental in the regulation of a variety of signaling pathways. The central feature of many receptor-mediated signaling events, in which extracellular agonists engage cognate receptors to activate signaling pathways, involves rapid alterations in protein phosphorylation. As yet, there is only limited evidence to suggest such a role for *O*-GlcNAc (6, 29, 30). Using a PMN model and fMLF as an extracellular stimulus, we have found that *O*-GlcNAc can be rapidly induced in a receptor-

dependent manner and consequently may act as a regulatory signal for downstream functional responses.

The receptor-derived signal for many functional responses is rapidly transduced, occurring over tens of seconds. Our data show changes in *O*-GlcNAc within this time frame. In conjunction with modified chemotactic responsiveness as a result of *O*-GlcNAc-inducing treatments, these data support a role for this post-translational modification in rapidly dynamic signal transduction. fMLF rapidly and robustly increased *O*-GlcNAc on a large number of proteins. The rate at which this modification was induced was evident in both immunofluorescence microscopy and immunoblots assays, in which fMLF caused an easily detected increase in CTD110.6 immunoreactivity within 30 s and 1-2 min, respectively. Furthermore, a number of proteins were modified by *O*-GlcNAc in response to fMLF. Our immunofluorescence analysis of the CTD110.6 label revealed that the immunoreactivity was predominantly cytoplasmic with the most concentrated labeling being associated with punctate structures in the cell. It is not clear whether this pattern is truly reflective of *O*-GlcNAc distribution or is in part a consequence of the methodology, because a variety of nucleus-associated proteins are known to be *O*-GlcNAcylated, including nuclear pore proteins and transcription factors (7). It may be that the large size of the CTD110.6 IgM prevents it from efficiently penetrating the nucleus, even in fixed cells. Nonetheless, the label we detected was shown to be specific for *O*-GlcNAc by competition with free GlcNAc, providing evidence for the validity of the assay in assessing the temporal dynamics of *O*-GlcNAcylation. In addition, $GlcNH_2$-induced increases in *O*-GlcNAc, in both immunoblot and immunofluorescence analyses, revealed similar results to those observed for fMLF. As a whole, these data provide sub-

stantive evidence of agonist-induced *O*-GlcNAcylation and represent the first evidence that such changes occur over short intervals.

We felt it essential to establish that exogenous $GlcNH_2$ would be metabolized by the HBP in a comparable time frame. Short-term $GlcNH_2$ treatments increased the levels of HBP metabolites, first $GlcNH_2$ 6-P and then UDP-GlcNAc. UDP-GlcNAc formation was dependent on the time and concentration of $GlcNH_2$ treatment, with treatments at high $GlcNH_2$ concentrations, 10 mM, significantly increasing UDP-GlcNAc within 30 min while lower concentrations required 60-120 min. Robust increases in *O*-GlcNAcylation occurred at 60-min $GlcNH_2$ treatment for most concentrations tested, although 10 mM $GlcNH_2$ increased *O*-GlcNAc within 30 min. Thus, when compared with the respective UDP-GlcNAc levels, there is a parallel between increases in UDP-GlcNAc and *O*-GlcNAcylation. Interestingly, at 100 μM $GlcNH_2$ increases in *O*-GlcNAcylation correlated with increases in UDP-GlcNAc of only 20-25%. These modest increases are likely relevant, although, because the protein acceptor specificity of OGT has been shown to be sensitive to small changes in UDP-GlcNAc (22). In addition, the observed levels of UDP-GlcNAc reflect net changes and not just UDP-GlcNAc synthesis and, thus, do not take into account its utilization, which may be significant as the length of $GlcNH_2$ treatment increases. Furthermore, the observed levels may not accurately reflect localized concentration changes that could occur in particular cellular compartments. This is relevant because cytosolic levels of UDP-GlcNAc are thought to be 10-20 times lower than endoplasmic reticulum/Golgi levels (50, 51).

We also combined $GlcNH_2$ and agonist treatments and found additive increases in *O*-GlcNAc, in particular for several high molecular weight proteins in the short duration

$GlcNH_2$ pre-treatments. These additive changes varied by protein, but clearly suggest that $GlcNH_2$ might modify agonist-induced signal transduction by altering the level of *O*-GlcNAcylation induced by the agonist itself. This also suggests how $GlcNH_2$ might alter agonist-induced functional responses such as chemotaxis.

By understanding the conditions under which UDP-GlcNAc and *O*-GlcNAc levels can be manipulated through HBP metabolism, we have begun to establish conditions for understanding how *O*-GlcNAc affects PMN function. The same conditions that altered HBP metabolism, UDP-GlcNAc formation and *O*-GlcNAcylation, also affected chemotactic but not respiratory burst activity. This suggests that protein *O*-GlcNAc modulates certain aspects of agonist-induced signaling. Furthermore, the diverse signaling pathways that lead from fMLF-associated receptor engagement to chemotactic and respiratory burst activity are different (34, 52-54).

In conclusion, our results support the theory that *O*-GlcNAc is a rapidly induced signal that operates in the context of physiological receptor-mediated signal transduction. The ubiquitous nature of this post-translational modification, the large numbers of target proteins that are either suggested or known to be modified by it, and its similarities to phosphorylation in modulating protein function suggest that it may play a general role in dynamic signaling in response to cellular stimulation.

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PROTEIN *O*-GlcNAc MODULATES MOTILITY-ASSOCIATED SIGNALING IN TERMEDIATES IN NEUTROPHILS

by

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Summary

The modification of Ser/Thr residues on cytoplasmic and nuclear proteins by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) is suggested to play a role in the regulation of a variety of signal transduction pathways. We have previously shown that glucosamine, a metabolic precursor of *O*-GlcNAcylation, increases *O*-GlcNAc and enhances motility in neutrophils. Here, we extend this correlation by showing that a mechanistically distinct means of increasing *O*-GlcNAc, achieved by inhibition of *O*-GlcNAc removal with *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate, increases basal cellular motility and directional migration induced by the chemoattractant formyl-methionine-leucine-phenylalanine. Furthermore, we demonstrate that *O*-GlcNAc modulates the activities of signaling intermediates known to regulate neutrophil movement. Glucosamine and *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate increase both the basal and formyl-methionine-leucine-phenylalanine-induced activity of a central mediator of cellular motility, the small GTPase Rac. Phosphoinositide-3 kinase, an important regulator of Rac activity and neutrophil motility, is shown to regulate the signaling pathway on which Glucosamine and *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate act. Rac is an important upstream regulatory element in p38 and p44/42 mitogen-activated protein kinase signaling in neutrophils, and these mitogen-activated protein kinases are implicated in chemotactic signal transduction. We show that glucosamine and *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate treatment increases p42/44 and p38 mitogen-activated protein kinase activities and that these increases are associated with activation of upstream mitogen-activated protein kinases. These data indicate that *O*-

GlcNAcylation is an important signaling element in neutrophils that modulates the activities of several critical signaling intermediates involved in the regulation of cellular movement.

Introduction

Numerous cytoplasmic and nuclear proteins are post-translationally modified by *O*-linked *N*-acetylglucosamine in β linkage to Ser/Thr residues (*O*-GlcNAc) (1, 2). The sugar donor for the *O*-GlcNAc transferase that catalyzes this modification is a product of glucose metabolism through the hexosamine biosynthesis pathway (HBP) (3). *O-GlcNAc transferase* activity is sensitive to changes in substrate availability such that increases in HBP flux, mediated either through glucose or glucosamine ($GlcNH_2$) administration, lead to increased levels of its substrate, UDP-*N*-acetylglucosamine, which then drives *O-GlcNAc transferase*-mediated *O*-GlcNAcylation (3-7). The dynamic nature of the *O*-GlcNAc moiety suggests that it may be functionally analogous to phosphorylation in influencing protein functions such as enzymatic activity, protein-protein interactions, and subcellular localization (1, 8-12). Supporting evidence for such a regulatory role comes from the variety of signaling pathways that are regulated by protein *O*-GlcNAc. For example, protein *O*-GlcNAc is associated with transcriptional regulation (13, 14), proteasome-mediated protein degradation (15), insulin signaling (16), and cellular stress signaling (17). Using a neutrophil (polymorphonuclear leukocyte or PMN) model, we recently demonstrated that rapid protein *O*-GlcNAcylation could occur in response to agonist stimulation through a cell surface receptor-mediated mechanism (18). These data pro-

vided further support for the concept that *O*-GlcNAc may function in a highly dynamic regulatory capacity analogous to protein phosphorylation.

PMNs are a vital component of the innate immune response to infection and tissue damage. Their effectiveness in this capacity is dependent on the activation of actin-based cytoskeletal machinery that allows directed migration toward a focus of inflammation. Lipid kinase, protein kinase, and small G protein activities are central mediators of PMN motility; however, we have recently implicated substrate-driven *O*-GlcNAcylation as an additional modulator of this essential PMN function (18). Specifically, $GlcNH_2$-associated increases in *O*-GlcNAc were found to correlate with enhanced basal and formylated methionine-leucine-phenylalanine (fMLF)-induced motility.

The intracellular signals that mediate PMN motility involve a complex interconnected signaling network that includes phosphoinositide-3 kinases (PI3Ks), small GTP-binding proteins of the Rho family, and mitogen-activated protein kinases (MAPKs). The generation of PI3Kγ knockout mice and the use of PI3K-specific inhibitors have demonstrated an important role for PI3K and its lipid products in chemotaxis (19-22). Knockouts have also been generated for the small GTPases Rac1 and Rac2, and these mice also show significant deficiencies in PMN chemotaxis (23-26). Interestingly, Rac activation may be regulated in large part by a recently identified guanine nucleotide exchange factor, P-Rex1 (27). This Rac guanine nucleotide exchange factor is activated by either phosphatidylinositol phosphates or heterotrimeric $G_{\beta\gamma}$ subunits, thus providing a direct link between PI3K and Rac activation. PMNs lacking Rac are also deficient in p38 and p44/42 activation (23, 26, 28), which is significant because pharmacological MAPK inhibition has indicated that the activities of p38 and p44/42 are important in chemotactic

signaling (29-36). This may occur through MAPK-mediated regulation of MAPK-activated protein kinase (35, 37, 38); leukocyte-specific protein 1 (39-41), an actin-binding protein that modulates cell motility; and heat shock protein 27 (42-45), which modulates actin polymerization and cellular migration in a variety of cell types.

In light of our recent study correlating $GlcNH_2$-induced increases in *O*-GlcNAc with enhanced PMN motility (18), the goal of this study was to examine the impact of *O*-GlcNAc on motility-associated signal transduction. Importantly, we also sought to confirm that protein *O*-GlcNAcylation was indeed the mechanism through which $GlcNH_2$ alters PMN function by examining whether an alternate, unrelated means of increasing *O*-GlcNAc has effects similar to those seen with $GlcNH_2$. We considered this essential because $GlcNH_2$ may have metabolic effects unrelated to protein *O*-GlcNAcylation (3, 46-48). Therefore, we compared the effect of *O*-GlcNAcylation induced by $GlcNH_2$ to that induced by the neutral β-*N*-acetylglucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (PugNAc) (49). By using these mechanistically unrelated methods of manipulating the enzymes responsible for *O*-GlcNAc cycling, we demonstrate that *O*-GlcNAc modulates neutrophil motility. In accordance with its effects on motility, we show that protein *O*-GlcNAc increases the amount of GTP-bound, or activated, Rac. Furthermore, *O*-GlcNAc-associated changes in cell motility are shown to be wortmannin sensitive. Increases in *O*-GlcNAc are also associated with increased activity of the MAPKs p38 and p44/42, and of the mitogen-activated protein kinase kinases (MKKs) MKK3/6 and mitogen-activated protein kinase extracellular signal-regulated kinases 1 and 2 (MEK1/2). In addition to confirming that protein *O*-GlcNAcylation modulates motility and motility-associated signaling, our data

support the idea that *O*-GlcNAc is an important signaling mechanism in PMNs akin to phosphorylation. Furthermore, our results are of general interest because they define a novel axis of *O*-GlcNAc-modulated signal transduction that involves Rac/PI3K/MAPK, each of which is an important signaling intermediate in a variety of systems and cellular processes.

Experimental Procedures

Materials

PugNAc was purchased from Carbogen. Wortmannin and fMLF were purchased from Calbiochem. GTPγS and GDP were from Upstate. Hank's buffered salt solution was purchased from Cellgro. Anti-actin antibody was purchased from Abcam. Anti-rabbit IgG and anti-mouse IgG were from Bio-Rad. All other reagents and chemicals were from Sigma unless otherwise indicated.

PMN Isolation

Whole blood from volunteers was obtained by venipuncture and layered onto a double discontinuous gradient formed with equal volumes of Histopaque-1077 (Sigma) over Histopaque-1119 (Sigma) (50). The blood was centrifuged at 700 x *g* for 30 min. Granulocytes were collected from the 1077/1119 interface and washed in Hank's buffered salt solution buffered with 10 mM HEPES, pH 7.4. Contaminating red blood cells were lysed by incubation in 0.15 M NH_4Cl/1 mM $KHCO_3$/0.1 mM EDTA for 5 min at 37 °C. The granulocytes were then washed twice in Hank's buffered salt solution and resus-

pended in Hank's buffered salt solution. Cell viability was assessed through Trypan Blue (Sigma) exclusion.

Immunoblotting

For protein *O*-GlcNAc immunoblots 2 x 10^6 PMNs were treated as indicated, and directly lysed in 5X sample buffer (0.3 M Tris·HCl/5% SDS/50% glycerol/0.025% bromophenol blue/5% mercaptoethanol), and boiled for 5 min. 7.5 x 10^5 cell equivalents per lane (~60 μg protein) were separated by SDS-PAGE (51) and transferred to Immobilon-P (Millipore). Immunoblotting was performed using a rapid immunodetection method for Immobilon-P (Millipore Tech Note TN051). Briefly, the membranes were equilibrated in methanol and air dried. The dry membrane was incubated with a 1:1000 dilution of anti-*O*-GlcNAc antibody CTD110.6 (52) (a kind gift of M. A. Accavitti, the University of Alabama at Birmingham) in 1% casein/phosphate-buffered saline (Pierce) with 0.01% Tween 20 for 2 h and then washed three times in phosphate-buffered saline. The membrane was then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM (Calbiochem) in 1% casein/ phosphate-buffered saline with 0.01% Tween 20 for 1 h. After further washing in phosphate-buffered saline the immunoblots were developed with enhanced chemiluminescence (SuperSignal West Pico; Pierce). For Rac and Ras immunoblots, the affinity-purified products from the Rac- and Ras-GTP assays and the total input lysates (see below) were separated by SDS-PAGE, transferred to Immobilon-P, and then immunoblotted with Rac-1 and Ras-specific antibodies (Upstate) according to the antibody manufacturer's directions. The immunoblots were visualized using enhanced chemiluminescence. The activities of p38, p44/42, MKK3/6, and

MEK1/2 were assessed by phosphorylation state-specific antibodies (Cell Signaling Technology). 2 x 10^6 PMNs were treated as indicated and then directly lysed in 5X sample buffer followed by boiling for 5 min. 7.5 x 10^5 cell equivalents per lane (~60 μg protein) were separated by SDS-PAGE and transferred to Immobilon-P. Immunoblotting was performed according to the antibody manufacturer's directions and visualized by enhanced chemiluminescence.

Motility Assays

2 x 10^5 PMNs were pre-treated as indicated, washed, resuspended in Dulbecco's modification of Eagle's medium /5% bovine serum albumin, and then placed in the upper filter plate of a 96-well MultiScreen-MIC chemotaxis plate (Millipore) with 3-μm membrane pores. The lower receiver plate contained Dulbecco's modification of Eagle's medium and 100 μM fMLF where indicated. Basal motility was assessed in the absence of fMLF. The assays were performed in a 37 °C incubator for 45 min. The filter plate was then carefully removed, and migrating cells were counted by microscopic examination of receiver plates (53).

Rac and Ras Activity Assays

Rac and Ras activities were assessed by the affinity capture of Rac- and Ras-GTP (54, 55). 10^7 PMNs were treated as indicated and then lysed by the addition of 5X Mg^{2+} lysis/wash buffer (125 mM HEPES, pH 7.5/750 mM NaCl/5% Igepal CA-630/50 mM $MgCl_2$/5 mM EDTA/10% glycerol). The lysates were cleared of insoluble debris by spin-

ning them at 14,000 x *g* for 10 min at 4 °C. ~30 μg of the resulting supernatant were mixed with 5X sample buffer, boiled for 5 min, and then set aside for determination of total (input) Rac or Ras by SDS-PAGE and immunoblot analysis as described above. The remainder of each supernatant was incubated with 5 μg of an agarose conjugate of either p21-binding domain of p21-activated kinase 1 (PAK-1 PBD) or Ras-binding domain of Raf-1 (Raf-1-RBD) (Upstate) for Rac- and Ras-GTP assays, respectively, for 1 h at 4 °C with gentle rocking. The beads were then washed three times with 1X Mg^{2+} lysis/wash buffer and finally resuspended in 2X sample buffer and boiled. The resulting affinity-purified products, active Rac or Ras, were then analyzed by SDS-PAGE and immunoblot as described above. GTPγS- and GDP-loaded positive and negative controls, respectively, were prepared as described above, except that before the addition of the beads to the supernatant, the supernatants were first incubated with either 100 μM GTPγS or 1 mM GDP for 30 min at 30 °C with gentle agitation, followed by the addition of 60 mM $MgCl_2$ to stop the loading.

Statistics

All graphical data are represented as mean values with error bars of ±2 standard errors from the mean. Significance was assessed with the Student's *t* test.

Results

PugNAc Increases Protein O-*GlcNAc and PMN Motility*

PugNAc is a widely used pharmacological inhibitor of the enzyme responsible for the removal of *O*-GlcNAc residues, neutral β-*N*-acetylglucosaminidase (49). This inhibi-

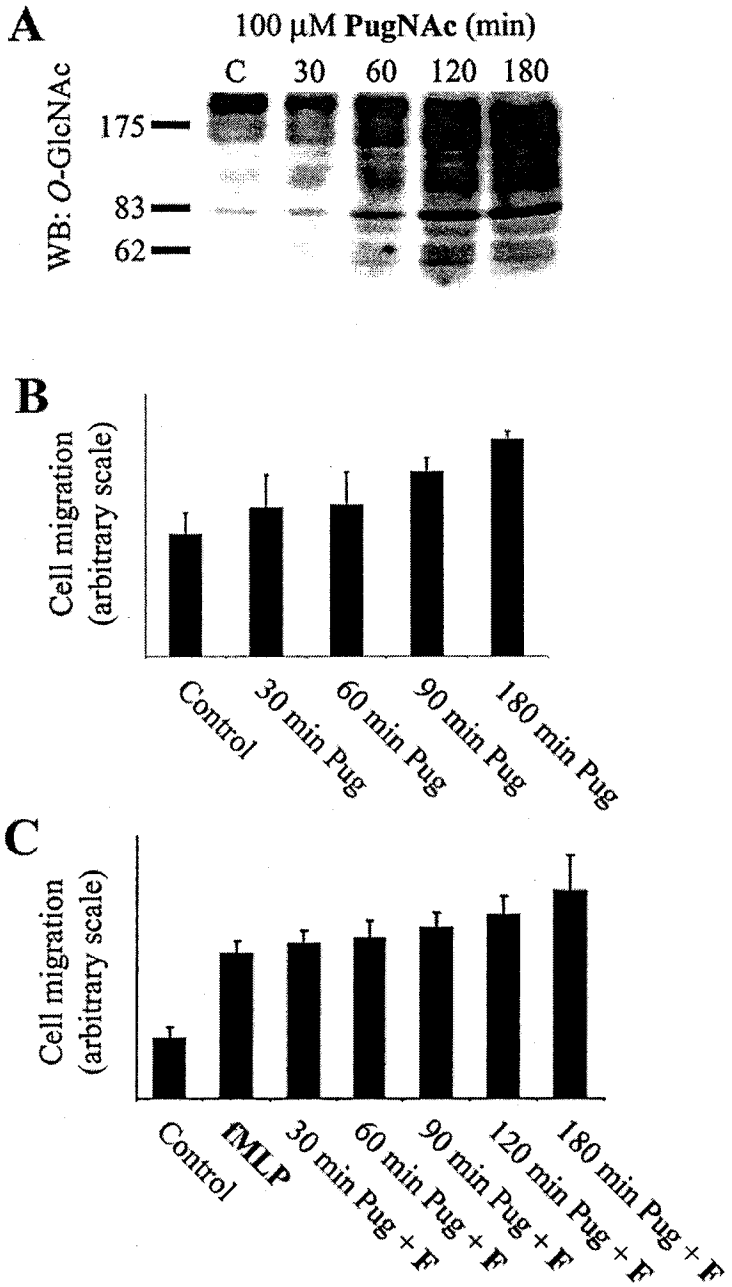
tor has proven to be a valuable tool for increasing protein *O*-GlcNAc in variety of systems (16, 49, 56, 57). As we have previously shown that $GlcNH_2$ effectively drives protein *O*-GlcNAcylation in PMNs through a substrate-driven, or metabolic, mechanism involving the HBP (18), we assessed whether PugNAc could serve as an alternative and complementary means of increasing *O*-GlcNAc levels.

Treatment with 100 μM PugNAc for selected times significantly increased protein-associated *O*-GlcNAc as determined by immunoblot analyses of whole cell extracts with the *O*-GlcNAc-specific antibody CTD110.6 (Fig. 1*A*). A variety of proteins with molecular weights above ~50 kDa displayed increased *O*-GlcNAc levels that were proportional to the duration of PugNAc treatment, beginning within 30 min and increasing to maximal levels of *O*-GlcNAcylation by 2 h. Interestingly, the pattern of *O*-GlcNAcylation was grossly similar to that induced by $GlcNH_2$ (18). There were no changes to cell viability, as assessed by Trypan Blue exclusion, at any of the time points examined (data not shown).

These results suggest a rapid rate of intrinsic *O*-GlcNAc cycling, providing further evidence of the highly dynamic nature of this post-translational modification in PMNs, and indicate that at least some of the same proteins may be *O*-GlcNAcylated in response to both $GlcNH_2$ and PugNAc treatments. Furthermore, the findings indicate that PugNAc can serve as an effective tool for rapidly inducing increases in *O*-GlcNAc and should be useful therefore for furthering our investigation of *O*-GlcNAc-associated changes in PMN motility.

As mentioned above, we have previously found that $GlcNH_2$ pretreatment increases basal (in the absence of chemoattractant) and fMLF induced PMN migration in

FIG. 1. ***O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate increases protein protein *O*-linked β-*N*-acetylglucosamine and polymorphonuclear leukocyte motility.** *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate was used to inhibit neutral β-*N*-acetylglucosaminidase activity (thereby preventing the enzymatic removal *O*-linked β-*N*-acetylglucosamine) and thus increase protein *O*-linked β-*N*-acetylglucosamine levels. *A*, immunoblots of whole cell lysates were performed using the anti- *O*-linked β-*N*-acetylglucosamine antibody CTD110.6. Polymorphonuclear leukocytes were left untreated (*C*) or were treated with 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*) for 30, 60, 120, or 180 min. WB, Western blot. *O*-GlcNAc, *O*-linked β-*N*-acetylglucosamine. *B*, basal polymorphonuclear leukocyte motility was assessed in the absence of chemotactic factor in Boyden chamber-type migration assays ($n \geq 3$ for all conditions). Polymorphonuclear leukocytes were left untreated (*Control*) or were treated with 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*Pug*) for 30, 60, 90, or 180 min. Significant ($p < 0.05$ as evaluated by Student's *t* test) increases in motility were observed for 90 and 180 min of *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate treatment when compared to control. *C*, chemotaxis was assessed in Boyden chamber-type migration assays ($n \geq 3$ for all conditions). Polymorphonuclear leukocytes were not pre-treated (*Control* and *fMLP*) or were pre-treated with 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*Pug*) for 30, 60, 90, 120, or 180 min. 100 nM formylated methionine-leucine-phenylalanine (*fMLP* and *F*) was used to initiate chemotaxis in the *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate pre-treated samples and the sample labeled *fMLP*. The control sample was not stimulated with chemotactic factor. Significant ($p<0.05$ as evaluated by Student's t-test) increases in motility were observed for 90, 120, and 180 min of *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate pre-treatment when compared formylated methionine-leucine-phenylalanine alone (*fMLP* without pre-treatment).



Boyden chamber-type assays and that such increases correlate with increases in protein *O*-GlcNAc (18). Having established that PugNAc increases *O*-GlcNAc levels, we sought to test whether PugNAc similarly increases PMN migration. Cells were pre-treated with 100 μM PugNAc for various durations and were then assayed for cell migration using simple 45-min Boyden chamber-type assays. PugNAc increased basal cell motility (in the absence of chemoattractant) in a time-dependent manner (Fig. 1*B*). Changes in basal motility were significant ($p < 0.05$) at 90 and 180 min of pretreatment. In assays of fMLF-associated chemotaxis (100 nM fMLF), 100 μM PugNAc pre-treatments of 90, 120, and 180 min were associated with significant increases (over the levels induced by fMLF without pre-treatment, $p < 0.05$ where $n \geq 6$ for all conditions) of 17%, 26%, and 42%, respectively, in cell migration (Fig. 1*C*). Thus the effects of PugNAc treatment are grossly similar in terms of basal motility and chemotaxis to the aforementioned results obtained using $GlcNH_2$ (18). The finding that two unrelated mechanisms of inducing protein *O*-GlcNAc similarly enhance motility strongly suggests that *O*-GlcNAc modulates neutrophil physiology.

PugNAc and $GlcNH_2$ Increase Both Basal and fMLF-induced Rac Activity in Rac-GTP-Specific Assays

We next sought to determine how protein *O*-GlcNAc might influence cell motility by examining some of the key facets of the signaling system that is thought to regulate the complex process of cellular movement. The small GTPase Rac is a well described regulatory component in chemotactic signaling that appears to have a central role in both establishing polarity and regulating the actin cytoskeleton (23-26, 58).

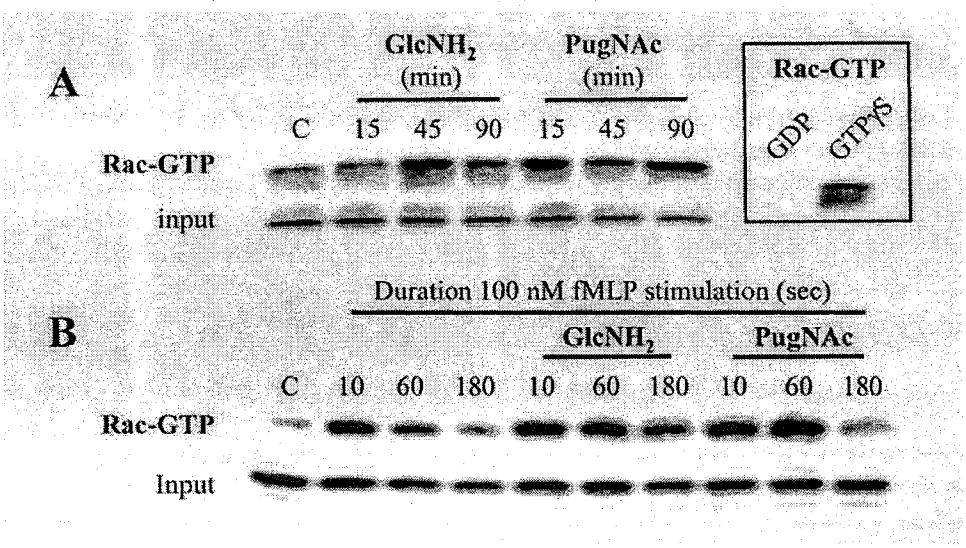


FIG. 2. ***O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate and glucosamine increase both basal and formylated methionine-leucine-phenylalanine-induced Rac activity.** Active GTP-bound Rac was assessed by affinity purification using the p21 binding domain of p21-activated kinase 1. The specificity of this assay for GTP-bound Rac was established by the *in vitro* loading of Rac with GTPγS or GDP prior to affinity purification (*inset*). *A*, basal Rac activity in polymorphonuclear leukocytes treated with glucosamine (*$GlcNH_2$*) or *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*). polymorphonuclear leukocytes were left untreated (*C*) or were treated with 10 mM glucosamine or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate for 15, 45, or 90 min. Total levels of Rac in the pre-assay lysates are indicated as *input*. *B*, chemoattractant-induced Rac activity in polymorphonuclear leukocytes pre-treated with glucosamine (*$GlcNH_2$*) or *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*). Polymorphonuclear leukocytes were not pre-treated (*C* and the adjacent three lanes labeled *10*, *60*, and *180* that are without *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate or glucosamine labels) or were pre-treated with 10 mM glucosamine or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate for 90 min. Rac-GTP was assessed after stimulating the cells with 100 nM formylated methionine-leucine-phenylalanine for 10, 60, and 180 s. The control sample (*C*) was not stimulated with chemotactic factor. Total levels of Rac in the pre-assay lysates are indicated as *input*.

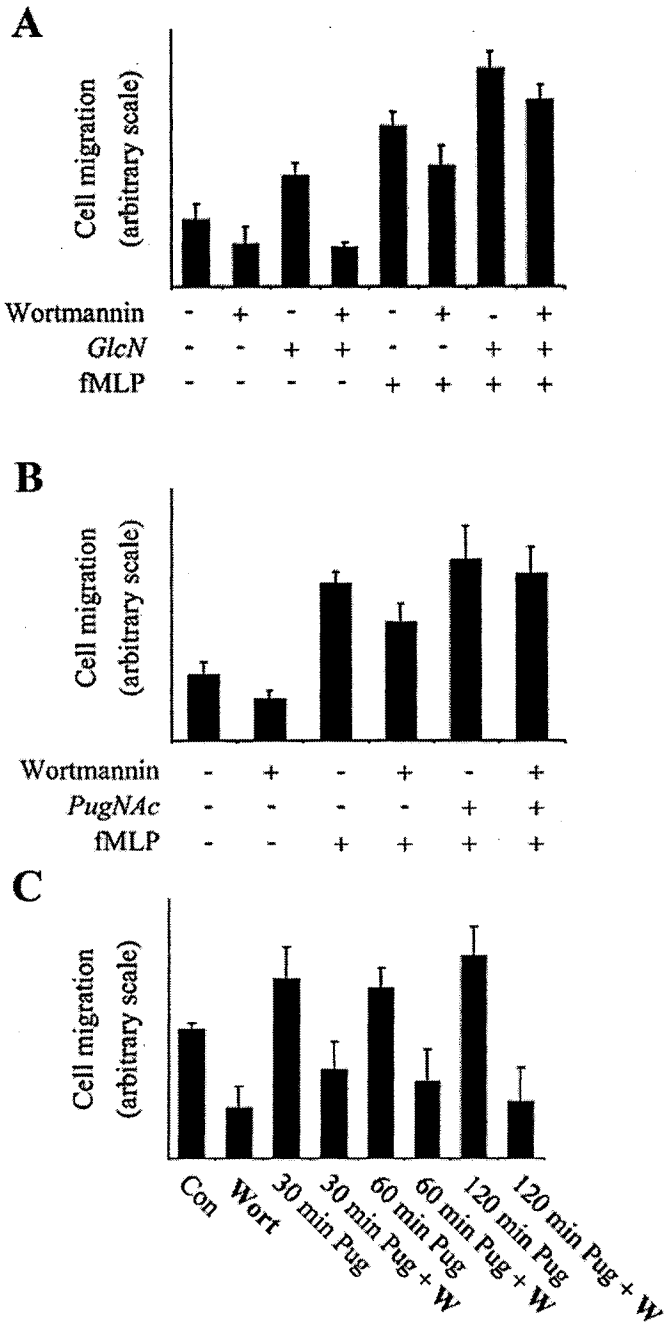
We examined whether PugNAc and $GlcNH_2$, both of which we had now established are modulators of motility, affected Rac1 activity. Active, GTP-bound Rac was assessed in Rac immunoblots following affinity capture with p21-binding domain of p21-activated kinase 1 (54). Using this method, investigators have shown that Rac-GTP levels rapidly and transiently increase above resting levels when PMNs are stimulated with the chemoattractant fMLF (25, 54). 100 μM PugNAc and 10 mM $GlcNH_2$ increased basal Rac activity even in the absence of fMLF (Fig. 2*A*). We then assessed the effect of 100 μM PugNAc and 10 mM $GlcNH_2$ on the activation of Rac induced by 10, 60, and 180 s of stimulation with 100 nM fMLF (Fig. 2*B*). $GlcNH_2$ and PugNAc pre-treatment markedly enhanced fMLF-induced Rac activation over at least the first 60 s of stimulation. These results suggest that protein *O*-GlcNAcylation might modulate cell motility in a Rac-dependent manner.

Wortmannin Inhibits PugNAc- and $GlcNH_2$-associated increases in Basal PMN Motility

Rac activation is closely tied to the lipid kinase activity of PI3K (25, 27). In fact, these signaling intermediates are thought to function together in a critical positive feedback loop that generates signal amplification at the leading edge of migrating cell (59, 60). We were therefore interested in assessing whether PI3K played a role in *O*-GlcNAc-associated increases in PMN motility.

We initially examined the effect of PI3K inhibition on $GlcNH_2$-associated increases in basal and fMLF-induced migration (Fig. 3*A*). PMNs were incubated with 250 nM wortmannin, which has been used extensively as an inhibitor of PI3K activity in

FIG. 3. **Wortmannin inhibits *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate- and glucosamine-associated increases in polymorphonuclear leukocyte motility.** Wortmannin was used to inhibit phosphoinositide-3 kinase and assess whether *O*-linked β-*N*-acetylglucosamine-mediated changes in polymorphonuclear leukocyte motility depend on phosphoinositide-3 kinase activity. Motility and migration were assessed in Boyden chamber-type migration assays ($n \geq 3$ for all conditions). *A*, the affect of wortmannin on glucosamine-associated changes in basal and chemoattractant-induced motility. Polymorphonuclear leukocytes were pre-treated with 250 nM wortmannin and/or 10 mM glucosamine (*$GlcNH_2$*) as indicated (untreated samples are indicated by – and treated samples by +). The glucosamine pre-treatments were for 60 min, and when they were concurrent with wortmannin pre-treatment, the wortmannin was added 15 min before the glucosamine. The wortmannin alone samples (without glucosamine) were treated with inhibitor for 75 min. The samples were assayed for basal motility (the absence of formylated methionine-leucine-phenylalanine is indicated as –) or chemotaxis in response to 100 nM formylated methionine-leucine-phenylalanine (*fMLP*) (indicated as +). *B*, the affect of wortmannin on *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate-associated changes in chemoattractant-induced motility. Polymorphonuclear leukocytes were pre-treated with 250 nM wortmannin and/or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*) as indicated (untreated samples are indicated by – and treated samples by +). The *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate pre-treatments were for 60 min, and when they were concurrent with wortmannin pre-treatment, the wortmannin was added 15 min before the *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate. The wortmannin alone samples (without *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate) were treated with inhibitor for 75 min. The samples were assayed for chemotaxis in response to 100 nM formylated methionine-leucine-phenylalanine (indicated as *fMLP* and +). *C*, the affect of wortmannin on *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate-associated changes in basal motility. Polymorphonuclear leukocytes were treated with 250 nM wortmannin (*Wort* and *+W*) and/or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*Pug*). When it was concurrent with wortmannin treatment, the wortmannin was added 15 min before the *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate. The *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate treatments were for 30, 60, or 120 min. The control sample (*Con*) was untreated, and the wortmannin alone sample (*Wort*) was treated with inhibitor for 45 min. The samples were assayed for migration in the absence of chemoattractant.



PMNs (21, 22), beginning 15 min before the addition of 10 mM $GlcNH_2$. The cells were then incubated with both wortmannin and $GlcNH_2$ (or with buffer alone for the control samples) for 60 min before being assessed for migration in simple 45-min Boyden chamber-type assays in either the absence (basal motility) or the presence (chemotaxis) of 100 nM fMLF. Wortmannin treatment did not result in changes to cell viability, as assessed by Trypan Blue exclusion (data not shown). Wortmannin effectively blunted basal PMN motility in the control and completely blocked the increase seen with $GlcNH_2$. As other studies have shown, wortmannin was only moderately effective at reducing fMLF-induced chemotaxis (61). This finding indicates that wortmannin-insensitive pathways are important in neutrophil chemotaxis. This indication is supported by the fact that PI3Kγ deficient murine PMNs still display chemotaxis in response to fMLF despite the fact that their ability to produce phosphatidylinositol 3,4,5,-triphosphate (PIP3) is severely compromised (19, 20). Interestingly, $GlcNH_2$ and wortmannin together resulted in a level of response to fMLF that was comparable with the level of response to fMLF alone, although less than that seen when only fMLF and $GlcNH_2$ were present.

Next, we examined whether wortmannin could inhibit PugNAc-associated changes in PMN motility. The cells were treated as detailed above, except that 100 μM of PugNAc were used instead of $GlcNH_2$. PugNAc, like $GlcNH_2$, produced a level of response to fMLF in the presence of wortmannin that was comparable with the level of response produced by fMLF alone (Fig. 3*B*). In the case of basal motility, wortmannin was effective at reducing the increases associated with 30, 60, and 120 min of PugNAc prêtreatment to control levels of inhibition (wortmannin was added 15 min before each PugNAc treatment and remained for the entire duration of those treatments) (Fig. 3*C*). These

data suggest that *O*-GlcNAc is acting to increase basal motility through a pathway that is PI3K dependent.

$GlcNH_2$ and PugNAc Increase the Rate of fMLF-induced MAPK and MKK Activation

The MAPK signaling cascade consists of a tripartite signaling hierarchy composed of a MAPK kinase kinase, MKK, and MAPK, with small G proteins of the Ras family often acting as upstream regulators of MAPK kinase kinase (62). For example, the interaction of Ras with Raf allows Raf to activate MEK1/2, which then in turn activates p44/42 (63, 64). Rac activity is required for p38 phosphorylation in fMLF-stimulated PMNs (23, 26). The pathway from Rac to p38 is not clear but likely involves MKK3 and an undefined MAPK kinase kinase (65). Rac, through an unknown mechanism, also appears to be a critical necessary regulator of p44/p42 activation in PMNs (23,26,28). The activities of p38 and p44/42 have been found to be important for PMN chemotactic signaling (29-36).

We assessed MAPK signaling following pre-treatment with $GlcNH_2$ and PugNAc. MAPK and MKK activation are conveniently measured by immunoblot analysis using phosphorylation state-specific antibodies. PMNs were pre-treated with 10 mM $GlcNH_2$ for either 30 or 90 min and then stimulated with 100 nM fMLF for 30, 60, or 90 s (Fig. 4). The $GlcNH_2$ pre-treated groups displayed faster rates of MAPK phosphorylation and hence of activation. This increase was apparent for MKK3/6-p38 and MEK1/2-p44/42. We did not observe fMLF-induced Jun kinase activation, and $GlcNH_2$ pre-treatment did not alter this finding (data not shown). Similar experiments were carried out using 100 μM PugNAc (Fig. 5). The PugNAc-pre-treated groups also displayed faster rates of

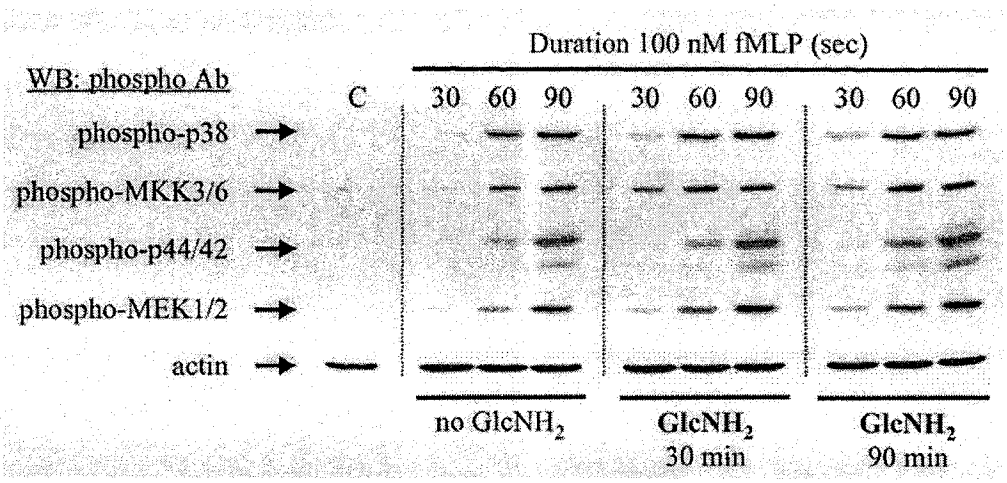


FIG. 4. **Glucosamine increases the rate of formylated methionine-leucine-phenylalanine-induced mitogen-activated protein kinase and mitogen-activated protein kinase kinase activation.** Mitogen-activated protein kinase and mitogen-activated protein kinase kinase activities from whole cell lysates were assessed by immunoblotting with the indicated phospho-specific antibodies. The control sample (*C*) was not pre-treated and was unstimulated. The cells were either not pre-treated with glucosamine (*no $GlcNH_2$*) or were pre-treated with 10 mM glucosamine (*$GlcNH_2$*) for 30 or 90 min. Mitogen-activated protein kinase activity was assessed at 30, 60, or 90 sec of stimulation with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*). Actin (*actin*) was used to verify equal sample loading. *WB*, Western blot. *Phospho-p38* and *phospho-p44/42*, phosphorylated forms of mitogen-activated protein kinases. *Phospho-MKK3/6*, mitogen-activated protein kinase kinase 3/6, and *phospho-MEK1/2*, extracellular-regulated kinase kinase 1/2, phosphorylated forms of mitogen-activated protein kinase kinases.

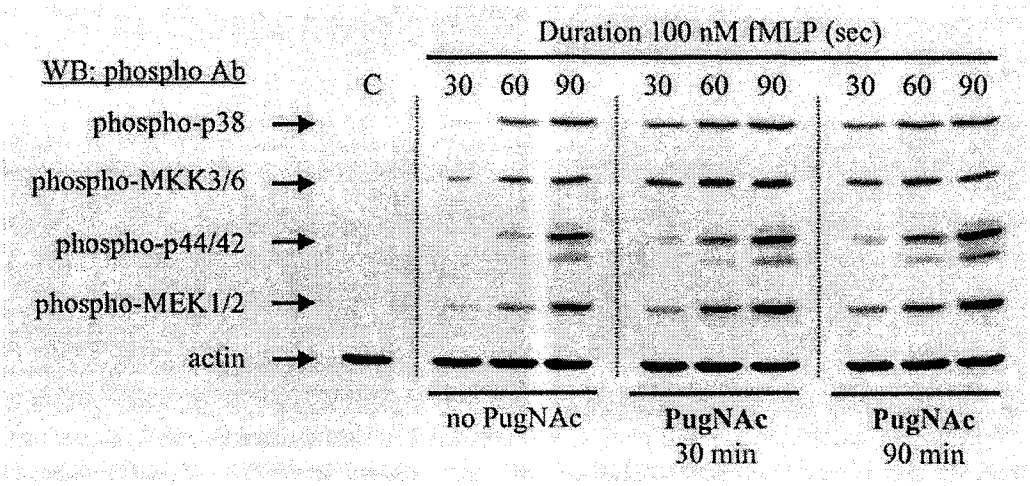


FIG. 5. ***O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate increases the rate of formylated methionine-leucine-phenylalanine-induced mitogen-activated protein kinase and mitogen-activated protein kinase kinase activation.** Mitogen-activated protein kinase and mitogen-activated protein kinase kinase activities from whole cell lysates were assessed by immunoblotting with the indicated phospho-specific antibodies. The control sample (*C*) was not pre-treated and was unstimulated. The cells were either not pre-treated with *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*no PugNAc*) or were pre-treated with 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*) for 30 or 90 min. Mitogen-activated protein kinase activity was assessed at 30, 60, or 90 sec of stimulation with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*). Actin (*actin*) was used to verify equal sample loading. *WB*, Western blot. *Phospho-p38* and *phospho-p44/42*, phosphorylated forms of mitogen-activated protein kinases. *Phospho-MKK3/6*, mitogen-activated protein kinase kinase 3/6, and *phospho-MEK1/2*, extracellular-regulated kinase kinase 1/2, phosphorylated forms of mitogen-activated protein kinase kinases.

MAPK activation. These data suggest that *O*-GlcNAcylation positively modulates MAPK activity and, due to the signaling link between Rac and MAPKs, also support the finding that *O*-GlcNAcylation positively modulates Rac activation.

$GlcNH_2$ and PugNAc Increase Basal Levels of Ras-GTP But Have Little Effect on fMLF-induced Ras Activity

We found that *O*-GlcNAcylation clearly influences MEK1/2-p44/42 signaling. Although fMLF induces p44/42 MAPK activity in a Rac-dependent manner in PMNs (23,26,28), the archetypal upstream G protein regulator of its activity under most circumstances is Ras and not Rac (62-64). We therefore thought that it was important to evaluate whether PugNAc or $GlcNH_2$ could affect Ras activity. Active, GTP-bound Ras was assessed in Ras immunoblots following affinity capture with Ras-binding domain of Raf-1 (55). 100 μM PugNAc and 10 mM $GlcNH_2$ increased basal Ras activity but had little effect on fMLF-induced activity (Fig. 6). These data suggest that Rac is a more likely candidate than Ras is for modulating the protein *O*-GlcNAc-associated changes in p44/42 MAPK activity that are induced by chemoattractant. It should be noted, though, that under some circumstances there is a considerable amount of cross talk between Rac and Ras (62, 66-68), leaving open the potential for *O*-GlcNAc to alter Rac activity through Ras or for Rac to cooperate with Ras in signaling p44/42 activation.

Discussion

Although changes in *O*-GlcNAcylation have been implicated in the pathogenesis of certain disease states (for example, diabetes mellitus (5, 16, 69, 70) and Alzheimer dis

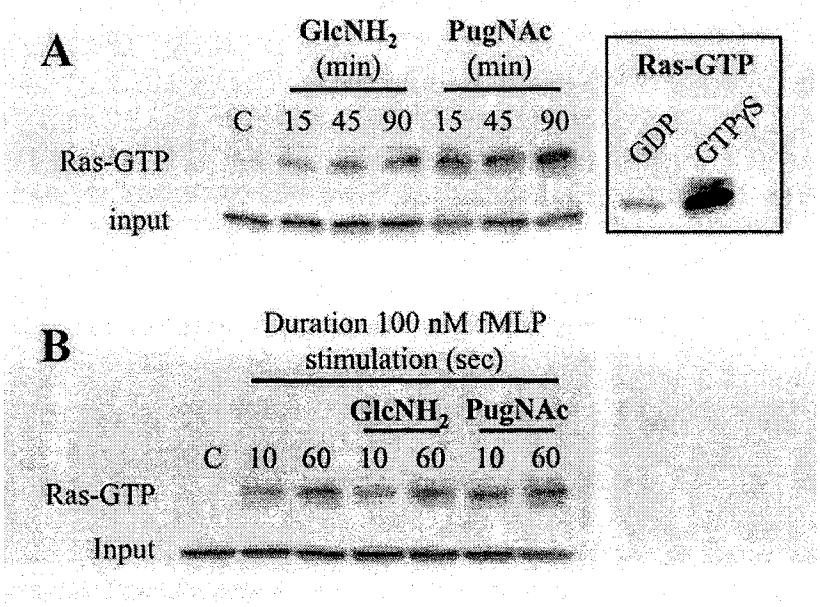


FIG. 6. **Glucosamine and *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate increase basal levels of Ras-GTP but have little effect on formylated methionine-leucine-phenylalanine-induced Ras activity.** Active GTP-bound Ras was assessed by affinity purification using the Ras-binding domain of Raf-1. The specificity of this assay for GTP-bound Ras was established by the *in vitro* loading of Ras with GTPγS or GDP prior to affinity purification (*inset*). *A*, Basal Ras activity in polymorphonuclear leukocyte treated with glucosamine or *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate. Polymorphonuclear leukocytes were left untreated (*C*) or were treated with 10 mM glucosamine (*$GlcNH_2$*) or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*) for 15, 45, or 90 min. Total levels of Ras in the pre-assay lysates are indicated as *input*. *B*, Chemoattractant-induced Ras activity in polymorphonuclear leukocytes pre-treated with glucosamine or *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate. Polymorphonuclear leukocytes were not pre-treated (*C* and the adjacent two lanes labeled *10* and *60* that are without *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate or glucosamine labels) or were pre-treated with 10 mM glucosamine (*$GlcNH_2$*) or 100 μM *O*-(2-acetamido-2-deoxy-d-glucopyranosylidene)amino-*N*-phenylcarbamate (*PugNAc*) for 90 min. Ras-GTP was assessed after stimulating the cells with 100 nM formylated methionine-leucine-phenylalanine (*fMLF*) for 10 or 60 sec. The control sample (*C*) was not stimulated with chemotactic factor. Total levels of Ras in the pre-assay lysates are indicated as *input*.

ease (71, 72)), the broader role of this ubiquitous post-translational modification in normal cellular function is as yet largely undefined. Previously, we reported that *O*-GlcNAc could be rapidly induced in PMNs by an extracellular agonist in a receptor-dependent manner and by $GlcNH_2$ through a substrate-driven mechanism involving the HBP (18). In those studies we showed that $GlcNH_2$-induced *O*-GlcNAcylation was associated with increases in cellular motility and migration. These data suggested that *O*-GlcNAc might act as a dynamic regulatory signal that modulates PMN physiology. Here we demonstrate for the first time that *O*-GlcNAcylation modulates the activities of several critical PMN signaling intermediates involved in the regulation of cellular movement. To expand on and verify the observation that $GlcNH_2$-induced *O*-GlcNAcylation is associated with altered PMN motility, we assessed whether PugNAc, which increases *O*-GlcNAc levels independently of the HBP, could serve as an alternative and complementary means of increasing *O*-GlcNAc and motility in PMNs. PugNAc increased protein *O*-GlcNAc beginning within 30 min of treatment, indicating that *O*-GlcNAc cycling is rapid in PMNs. This is an important observation in itself because it provides additional evidence that protein *O*-GlcNAcylation is a highly dynamic process in these cells and because it further substantiates the concept that *O*-GlcNAc may be important to normal PMN physiology. It also indicates that signals that lead to a reduction in *neutral B-N-acetylglucosaminidase* activity (in response to agonist, for example) may be important means of inducing *O*-GlcNAcylation in these cells in addition to *O*-GlcNAc transferase-mediated mechanisms. Like $GlcNH_2$, PugNAc increased basal and chemoattractant-induced motility. This result solidifies the conclusion that *O*-GlcNAc modulates neutrophil motility. Although we might expect that there are differences in the specificity of these agents for *O*-GlcNAc

acceptor proteins (because they act through different mechanisms) and that these differences could result in differential *O*-GlcNAcylation, our data suggest that, in the case of motility-associated signaling, the same relevant proteins are being *O*-GlcNAcylated. This suggestion is supported by the observation that the overall patterns of *O*-GlcNAcylation induced by PugNAc and $GlcNH_2$ are grossly similar. The fact that two dissimilar *O*-GlcNAc-inducing treatments enhance motility strongly supports a role for *O*-GlcNAc as a regulatory mechanism in PMN motility.

We next sought to extend our physiological observations into the signaling pathways known to influence motility. The small GTPase Rac is an essential molecular switch in regulating actin assembly and motility in PMNs (23-26, 58). Therefore, we examined whether increased *O*-GlcNAc levels influenced Rac activity. Three types of Rac have been identified (73), although most studies have focused on the roles of Rac1 and Rac2. Rac1 is ubiquitously expressed, whereas Rac2 expression is restricted to hematopoietic cells (73). Although the precise roles of each of these isoforms remain largely undefined, recent evidence indicates that Rac2 may be the primary regulator of actin assembly and the molecular machinery associated with movement and that Rac1 may be important for chemoattractant-directed orientation and gradient detection (74). In addition, Rac1 and Rac2 appear to exhibit cross talk with one another, and their activities appear to be closely associated (75). This would explain why combined Rac1/Rac2 knockouts demonstrate a significantly worse chemotactic defect than single knockouts of either Rac1 or Rac2 do (23). Our data indicate that *O*-GlcNAcylation is associated with increases in both basal and chemoattractant-directed Rac1 activity and that $GlcNH_2$ and PugNAc are equally effective in their effects on Rac activity. Unfortunatel, we were un-

able to adequately assess Rac2 activity. Although the Rac2-specific antibodies we used readily detected Rac2 in the input lysates used for Rac-GTP affinity assays, they did not detect Rac2-GTP associated with p21-binding domain of p21-activated kinase 1 (data not shown). Thus, it remains unclear how Rac2 it is affected by *O*-GlcNAc. Interestingly, the effects of increased *O*-GlcNAcylation on Rac1 were more pronounced during fMLF-induced Rac activation. *O*-GlcNAc lengthened the active phase of Rac activation, maintaining maximal levels of Rac activity for at least 60 s as opposed to 10 s in the absence of PugNAc or $GlcNH_2$ pre-treatment. *O*-GlcNAc did not appear to increase the absolute level of Rac activation, though. These data suggest that *O*-GlcNAc acts at or upstream of Rac and represent the first example of *O*-GlcNAc-associated changes in Rac activity. However, a recent study has shown that increased flux through the HBP could lead to increased Rho activity, although a specific role for *O*-GlcNAcylation was not assessed (76).

PI3K is an important regulatory intermediate of PMN motility that is closely tied to Rac (25, 27). Type 1 PI3Ks can be activated by cell surface receptors to produce PIP3 (22), which acts as a membrane-associated docking site for pleckstrin homology domains (77). PI3K activity directs chemoattractant-induced Rac activation, although Rac participates in a positive feedback loop that further induces PI3K and PIP3 accumulation (59, 60). This amplifies the polarized asymmetry of PIP3 and polymerized actin at the leading edge, critical events in motility and directional migration. Exogenous PIP3 is capable of inducing migration and actin polymerization in a wortmannin- and Rho-family GTPase (presumably Rac)-dependent manner (78), indicating that in the absence of additional stimuli isolated PI3K and Rac activation may induce changes in cellular motility. Therefore, we examined the role of PI3K in mediating the effects of *O*-GlcNAcylation on mo-

tility. Wortmannin effectively blocked the ability of PugNAc and $GlcNH_2$ to induce changes in basal motility, or chemokinesis. The ability of wortmannin to effectively block fMLF-associated chemotaxis was reduced in $GlcNH_2$, and PugNAc- pre-treated PMNs. These data are consistent with the notion that *O*-GlcNAc-mediated changes in motility occur through a mechanism that requires PI3K activity; however, further studies are required to define more precisely the role of PI3K in *O*-GlcNAc-associated changes in motility.

MAPKs are downstream effectors in the signaling pathways that mediate motility. In PMNs, pharmacological inhibition of p38 and p44/42 MAPKs has been shown to decrease chemotaxis (29-36). Although the precise role of MAPK activation in regulating motility is unclear, both p38 and p44/42 phosphorylate and activate MAPK-activated protein kinase (35, 79), which in turn regulates leukocyte-specific protein 1 (39) and the small heat shock protein 27 (80). leukocyte-specific protein 1 and heat shock protein 27 are involved in the regulation of the actin cytoskeleton and motility (39, 41-45). We found that increases in *O*-GlcNAc lead to significant increases in the rate of MAPK cascade activation, with a slight increase in the maximal level of MAPK and MKK phosphorylation. Because Rac regulates the p38 and p44/42 signaling cascades in fMLF-stimulated PMNs, these increases in *O*-GlcNAc-associated MAPK activity are consistent with similarly increased levels of active Rac in response to *O*-GlcNAcylation. Ras, however, is the traditional and best described mediator of MAPK signaling with respect to p44/p42 (62-64). We found that, although Ras-GTP was not increased substantially with fMLF stimulation, its basal levels were elevated in association with increased *O*-GlcNAc

levels. This finding then leaves Rac as the stronger candidate for mediating *O*-GlcNAc-associated changes in MAPK activity.

Several studies, all performed under different cellular contexts, have recently demonstrated that increases in HBP flux can lead to increased MAPK activation (76, 81-84). In addition, HBP-driven changes in p44/p42 activation have been suggested to occur through a Rho GTPase-mediated mechanism in cultured rat aortic smooth muscle cells (76). Of further significance is the finding that high concentrations of glucose, potentially through the HBP, sensitize vascular smooth muscle cells to serum, inducing chemotaxis via pathways involving PI3K, the Ras super-family of GTPases, and p44/42 MAPK (increased phosphorylation of p38 was also observed but was not associated with glucose-mediated changes in migration) (84). Our data support a role for Rac (a Rho-family GTPase), PI3K, and the p38 and p44/42 MAPKs in *O*-GlcNAc-associated changes in PMN migration. The similarities among these varied studies indicate that there may be a common intermediate signaling mechanism that underlies *O*-GlcNAc-mediated increases in MAPK activity, small monomeric GTPase activity, and motility.

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DISCUSSION

O-GlcNAcylation is suggested to regulate protein function in a manner analogous to phosphorylation (91-94). This comparison is a significant because protein phosphorylation is well established as the archetypal regulatory mechanism for transducing extracellular signals into specific responses. An essential property of phosphorylation in performing this function is that it is a dynamic and highly inducible signal; rapidly induced phosphorylation cascades, resulting from agonist engagement of cognate cell surface receptors, are a defining feature of the response of a cell to its environment. Prior to our current set of studies, there was only limited evidence to suggest that *O*-GlcNAc could operate in a similar manner. There was some indication that *O*-GlcNAc was dynamic, but there were no clear data in support of its physiological induction by agonist (95-97).

A principal goal of this study was to examine the temporal dynamics of agonist-induced *O*-GlcNAcylation. We hypothesized that *O*-GlcNAc could be rapidly induced in response to stimulation with an extracellular agonist. Using a chemoattractant stimulus in the context of a neutrophil model, we have found support for our hypothesis and in doing so have discovered two important features of *O*-GlcNAc-associated signaling: we demonstrate for the first time that *O*-GlcNAc can be robustly induced by an extracellular stimulus in a receptor-dependent manner and that this induction can be very rapid.

These initial observations allowed us to extend our hypothesis: the *O*-GlcNAc signals generated in response to a stimulus must have functionally significant impacts on

cellular physiology and function. Using the same experimental model of chemoattractant-stimulated neutrophils, we examined whether two critical neutrophil outputs, respiratory burst activity and cellular motility, were affected by changes in *O*-GlcNAcylation. We have now demonstrated that *O*-GlcNAcylation selectively modulates neutrophil motility and migration. Many of the signaling intermediates that regulate motility are well defined in fMLF-stimulated neutrophils (25). This allowed us to first predict and later demonstrate that *O*-GlcNAc modulates some of these intermediates in parallel fashion to motility. From our data we predict that Rac is a central mediator of *O*-GlcNAc-associated changes in cellular motility. All of the data presented in this dissertation suggest that *O*-GlcNAc is an important regulatory modification that can be induced to alter signal transduction and cellular function. Because we have found that *O*-GlcNAcylation is a physiologically dynamic signal, our data support the contention that *O*-GlcNAcylation is analogous to phosphorylation.

The Temporal Dynamics of Agonist-induced *O*-GlcNAcylation

Evidence of Dynamic Agonist-induced O-*GlcNAcylation*

The extracellular signals that modulate many cellular activities are rapidly transduced inside a cell, sometimes occurring within tens of seconds (118-120). We have demonstrated that receptor-derived *O*-GlcNAc signals can be transduced in a similar time frame. In CTD110.6-specific immunoblots the chemoattractant fMLF was shown to rapidly and robustly increase *O*-GlcNAc on a large number of proteins. fMLF increased CTD110.6 immunoreactivity within 1 min of stimulation. Immunofluorescence analysis of the CTD110.6 label revealed predominantly cytoplasmic *O*-GlcNAcylation. The most

concentrated signal was associated with punctate structures in the cytoplasm. The signal was shown to be specific for *O*-GlcNAc by competition with free N-acetylglucosamine. In addition, $GlcNH_2$-induced increases in *O*-GlcNAc, in both immunoblot and immunofluorescence analyses, revealed results similar to those observed for fMLF. As a whole, these data provide substantive evidence of agonist-induced *O*-GlcNAcylation and represent the first evidence that such changes occur over short intervals.

Neutrophils Possess a Functional Hexosamine Biosynthesis Pathway

We also established that neutrophils possess a functional HBP that can produce the required sugar donor for *O*-GlcNAcylation. Short term $GlcNH_2$ treatments increased the levels of the HBP metabolites $GlcNH_2$ 6-P and UDP-GlcNAc. UDP-GlcNAc formation and increases in protein *O*-GlcNAc were dependent on the time and concentration of $GlcNH_2$ treatment. These data revealed a parallel relationship between increases in UDP-GlcNAc and *O*-GlcNAcylation. One of the most interesting findings of these studies was that increases in *O*-GlcNAcylation at 100 μM of $GlcNH_2$ treatment correlated with increases in UDP-GlcNAc of only 20-25%. These modest increases in substrate may be relevant though, as the protein acceptor specificity of OGT has been shown to be sensitive to small changes in UDP-GlcNAc (107). In addition, the observed levels of UDP-GlcNAc reflect net changes and not just UDP-GlcNAc synthesis and thus do not take into account its utilization, which may be significant as the length of $GlcNH_2$ treatment increases. Furthermore, the observed levels may not accurately reflect localized concentration changes that could occur in particular cellular compartments. This is relevant because cytosolic levels of UDP-GlcNAc are thought to be 10-20 times lower than endo-

plasmic reticulum/Golgi levels (121,122). Future studies should attempt to analyze cytosolic UDP-GlcNAc in isolation as it is possible that we may be underestimating compartmentalized changes in its levels. It may be possible to selectively permeate the plasma membrane without affecting the intracellular membranes that make up the endoplasmic reticulum/Golgi.

O-GlcNAcylation Modifies Neutrophil Motility

By understanding the conditions under which UDP-GlcNAc and *O*-GlcNAc levels can be manipulated through HBP metabolism, we have begun to establish conditions for understanding how *O*-GlcNAc affects PMN function. $GlcNH_2$ treatment increased basal and fMLF-induced neutrophil motility while having little effect on respiratory burst activity. Thus the same conditions that altered HBP metabolism, UDP-GlcNAc formation and *O*-GlcNAcylation, also affected chemotactic but not respiratory burst activity. This suggests that protein *O*-GlcNAc modulates certain aspects of agonist-induced signaling and not others. By combining $GlcNH_2$ and agonist treatments, we found evidence that $GlcNH_2$ might modify agonist-induced signal transduction by altering the level of *O*-GlcNAcylation induced by agonist alone. This finding sheds light on why $GlcNH_2$ might alter agonist-induced functional responses such as chemotaxis and suggests that fMLF-induced *O*-GlcNAcylation is a physiological mechanism in motility-associated signal transduction.

Why Are Changes in O-*GlcNAc Selective for Motility-associated Signaling Pathways?*

Many of the same signaling intermediates that are important in chemotactic signaling are also important in NADPH oxidase signaling (refer to the "Introduction " above, which details the major signaling intermediates associated with motility and NADPH oxidase activation), and it is unclear why respiratory burst activity is not affected by changes in *O*-GlcNAcylation.

One possibility is suggested by the differential role of Rac1 *versus* Rac2 in respiratory burst signaling. Rac2-deficient murine neutrophils display impaired NADPH oxidase activity, whereas Rac1-deficient PMNs do not do so (56). Both isoforms, however, are important in chemotactic signaling (37, 56). Thus it may be that *O*-GlcNAc affects Rac1 and not Rac2. Unfortunately, we were unable to establish a reliable assay for Rac2 activation, and we do not know how changes in *O*-GlcNAc affect its function. This is a problem that should be addressed in future experiments, as differential regulation of Rac isoform activity by *O*-GlcNAc would be a significant finding.

Another possibility is that our assay for respiratory burst activity was not suitable for detecting alterations in ROS production induced by $GlcNH_2$ treatment. We could attempt to resolve any potential problems by using PugNAc, which is chemically distinct from $GlcNH_2$ and works in a mechanistically different manner to induce changes in *O*-GlcNAcylation (109), or by using a different assay to detect ROS.

Finally, it may be that *O*-GlcNAcylation is restricted to specific cytoplasmic compartments by localized OGT activity. Therefore, the stimulation of OGT in one part of the cell and not in another could lead to the differential *O*-GlcNAcylation of a protein that is present in both parts. If the two cellular compartments to which this protein was

localized were specific for certain cellular functions that is, actin remodeling at the plasma membrane *versus* NADPH oxidase activation at granular/vesicular surface, then we could expect that localized *O*-GlcNAcylation could have different effects on these functions. For example, Rac is localized to different regions within neutrophils that are associated with different functions (123,124). Interestingly, Rac1 and Rac2 also display different subcellular localization (125). Future studies should address these possibilities. Performing immunofluorescence microscopy with OGT-specific antibodies may make it possible to identify whether OGT is differentially localized within the cell and whether it selectively co-localizes with certain signaling intermediates. In addition, it should be possible to affinity purify OGT from cellular lysates, again using OGT-specific antibodies, and examine the signaling intermediates with which it co-purifies.

PugNAc Increases O-*GlcNAc and Motility*

To verify the idea that *O*-GlcNAcylation is associated with altered PMN motility, we assessed whether PugNAc, which increases *O*-GlcNAc levels independently of the HBP (109), could serve as an alternative and complementary means of increasing *O*-GlcNAc and motility in PMNs. PugNAc was a strong inducer of protein *O*-GlcNAc. This provided additional evidence that protein *O*-GlcNAcylation is a dynamic process in these cells and further substantiates the concept that *O*-GlcNAc may be important to normal PMN physiology. The finding also indicates that signals that lead to a reduction in neutral β-*N*-acetylglucosaminidase activity (in response to agonist, for example) may be important means of inducing *O*-GlcNAcylation in these cells in addition to OGT-mediated mechanisms. Like $GlcNH_2$, PugNAc increased basal and chemoattractant-induced motil-

ity. It appears that the same relevant proteins are being *O*-GlcNAcylated by these mechanistically dissimilar treatments, as the overall patterns of *O*-GlcNAcylation induced by PugNAc and $GlcNH_2$ are grossly similar. Identifying these proteins should be the central focus of any future studies (see "Future Studies" below).

O-*GlcNAc Modifies Motility-associated Signaling Intermediates*

Changes in O-*GlcNAc modify Rac activity.* Rac is an essential regulatory intermediate in actin assembly and motility in PMNs (37, 54-56). Increases in *O*-GlcNAc levels, induced by either $GlcNH_2$ or PugNAc, are associated with increases in both basal and chemoattractant-directed Rac activity. *O*-GlcNAc lengthened the duration but not the absolute level of Rac activation. This suggests that *O*-GlcNAc acts at or upstream of Rac. It is important to note that two other studies have shown that HBP-dependent changes in certain signaling pathways are mediated by a Ras super-family GTPase (in one of these studies, the link was narrowed down to the Rho subfamily of small GTPases, of which Rac is a member) (126, 127). Ras-related GTPases may be a common target for *O*-GlcNAc-associated modulation of signal transduction.

MAPKs are downstream effectors in the signaling pathways that mediate motility (61-68). We found that increases in *O*-GlcNAc lead to significant increases in the rate of MAPK cascade activation. Because Rac regulates the p38 and p44/42 signaling cascades in fMLF-stimulated PMNs (37, 38, 54), these increases in *O*-GlcNAc-associated MAPK activity are consistent with similarly increased levels of active Rac in response to *O*-GlcNAcylation. Several studies have demonstrated that increases in HBP flux can lead to increased MAPK activation (126-130). Their results and those of our studies further sug-

gest that there may be a common intermediate signaling mechanism that underlies *O*-GlcNAc-mediated increases in MAPK and small monomeric GTPase activities. Additional studies will have to be carried out to determine the target of *O*-GlcNAcylation that interacts with these signaling intermediates.

The Physiological Context of Protein *O*-GlcNAcylation

Our data lead to a final question with respect to *O*-GlcNAc signaling: what is the physiological context in which *O*-GlcNAc has an important signaling role? Whether *O*-GlcNAc functions as a general signaling mechanism that operates under a diverse set of conditions (as phosphorylation does) or, instead, as a specialized signal in the context of specific conditions remains to be seen. The fact that the enzymes responsible for *O*-GlcNAc cycling are ubiquitously expressed suggests a more generalized function (97). However, these enzymes are also the products of single genes, which necessarily limits the regulatory diversity of the *O*-GlcNAc modification they catalyze (97). Unfortunately, there is little information with respect to the physiological induction of *O*-GlcNAcylation. Our findings seem to indicate that, at least in neutrophils, *O*-GlcNAcylation is a global response, with apparently the same proteins modified as a result of $GlcNH_2$, PugNAc, or agonist treatment. This suggests there is relatively little diversity in the induction of *O*-GlcNAcylation. Interestingly, when the *O*-GlcNAc-specific immunoblots obtained from $GlcNH_2$-, PugNAc-, or fMLF-induced neutrophils are compared with those obtained from completely different cell types that have been induced by various forms of stress, the protein patterns are very similar (114). This lack of diversity occur because only a single gene product, OGT, can catalyze *O*-GlcNAc formation. In the case of stress-induced *O*-

GlcNAcylation *versus* that induced by fMLF, it could be argued that fMLF, as a signal for inflammation, is in fact a stress-inducing agonist. Therefore, these different stimuli might lead to the same responses. Clearly, fMLF stimulates the activation and expression of several stress-associated proteins such as heat shock proteins (117). However, how do the other established regulatory activities of *O*-GlcNAc fit into this picture? For example, *O*-GlcNAc modifies and regulates the activities of transcription factors (87,88) and proteasomal machinery (113). It is possible that these activities are part of an overarching stress response that is regulated by *O*-GlcNAc-associated changes in protein expression and degradation. It will only be by comparing the data from a large and diverse group of studies that we will get some sense of the general or specific roles of this still elusive post-translational modification.

Future Studies

The Specific O-*GlcNAcylated Proteins That Modulate Motility-associated Signaling*

With respect to this dissertation, the single most important study for the future is one that addresses the mechanism through which changes in *O*-GlcNAcylation alter motility-associated signaling. On the basis of the facts that Rac is a central component of motility-associated signaling and that its activity is modulated by changes in *O*-GlcNAcylation, any future studies need to focus on the identity of the *O*-GlcNAc-modified protein(s) that modulate Rac signaling. This could be accomplished in a number of ways; however, two broad approaches should be utilized (the general features of these approaches have been characterized in several recent publications (131-133)).

Analysis of the O-*GlcNAc proteome for candidate effectors of Rac.* In one approach, the *O*-GlcNAc proteome should be analyzed for candidate proteins that are known to be involved in Rac signaling. This would involve first the enrichment and purification of *O*-GlcNAcylated proteins and then the identification of those proteins. It is difficult to affinity purify and identify such proteins. Good affinity purification methods have been lacking, as convenient methods of specifically identifying *O*-GlcNAc-associated terminal *N*-acetylglucosamine with high levels of sensitivity and low background have not been developed (132). In addition, the analysis of affinity purified proteins is cumbersome, requiring specialized equipment and techniques that are not easily adapted to all laboratories. Recently however, new methods of analyzing the *O*-GlcNAc proteome have emerged that appear to eliminate many of these problems (131).

Identifying O-*GlcNAcylated effectors of Rac directly.* The second approach should involve examining how signaling intermediates know to interact with Rac and determining whether any of those intermediates are *O*-GlcNAcylated. This would involve the affinity purification of a protein that interacts with Rac, directly or indirectly, and a determination of its *O*-GlcNAcylation state. Candidate proteins include PI3K and its regulatory subunit, p85; Rac GAPase-activating proteins and GEFs, of which there are several; and associated signaling pathways that influence Rac activity, such as Src family kinases (55). This approach is again complicated by the fact that methods of specifically identifying *O*-GlcNAc-associated terminal *N*-acetylglucosamine with high levels of sensitivity and low background have not been developed. For example, it is likely that CTD110.6

(134) does not detect all *O*-GlcNAcylated protein epitopes. The other widely used methods of detecting O-GlcNAcylated residues are also problematic (135). Galactosyltransferase-mediated labeling of terminal *O*-GlcNAcs with radioactive galactose is specific but not sensitive and thus requires high concentrations of any proteins of interest (135). As many signaling intermediates occur in low abundance, this limits the use of this technique unless sufficiently enriched samples can be prepared. Because of these issues, it will be important to use a variety of techniques to identify the *O*-GlcNAcylation state of any given protein of interest and to develop effective techniques of enriching for specific proteins.

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APPENDIX

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORM

UAB THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

The Institutional Review Board for Human Use (IRB) has an approved Multiple Project Assurance with the Department of Health and Human Services and is in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on January 1, 1999 and the approval period is for five years. The Assurance number is M-1149.

Principal Investigator:	MARCHASE, RICHARD B
Co-Investigator(s):	
Protocol Number:	**X031006014**
Protocol Title:	*Calcium Signaling in Human Neutrophils*

The IRB reviewed and approved the above named project on 3-30-04. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 3-30-04

Date IRB Approval Issued: 3-30-04

HIPAA Waiver Approved?: No

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Zachary Thomas Kneass

Graduate Program Cell Biology

Title of Dissertation Characterization of Protein *O*-GlcNAc in Neutrophils

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

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