Cloning and expression of bovine p47-phox and p67-phox
by Peggy Lee Ohmstede Bunger

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Molecular Biology
Montana State University

Abstract:
Neutrophils play an essential role in bovine cellular host defense, and compromised leukocyte function has been linked to the development of respiratory and mucosal infections. During the host defense process, neutrophils migrate into infected tissues where they become activated, resulting in the assembly of neutrophil membrane and cytosolic proteins to form a superoxide anion-generating complex known as the NADPH oxidase. Two of the essential cytosolic components of the NADPH oxidase are p47-phox and p67-phox. Currently, only the human and murine homologues of these proteins have been sequenced. Because of the important role neutrophils play in bovine host defense, I carried out studies to clone, sequence, and express bovine p47-phox and p67-phox. Using PCR cloning techniques and a bovine bone marrow cDNA library, I cloned both of these bovine NADPH oxidase cytosolic components. Comparison of the bovine sequences with those of the human and murine homologues showed that they were highly conserved, but also revealed important information regarding key structural features of p47-phox and p67-phox, including location of putative phosphorylation sites. Functional expression of bovine p67-phox and p67-phox showed that these proteins could substitute for the human proteins in reconstituting NADPH oxidase activity in a cell-free assay system, again demonstrating the high degree of conservation between human and bovine homologues. This study greatly contributes to our understanding of the potential structural/functional regions of p47-phox and p67-phox as well as gives us information that can be used to study the role of neutrophils in bovine inflammatory diseases.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Neutrophils play an essential role in bovine cellular host defense, and compromised leukocyte function has been linked to the development of respiratory and mucosal infections. During the host defense process, neutrophils migrate into infected tissues where they become activated, resulting in the assembly of neutrophil membrane and cytosolic proteins to form a superoxide anion-generating complex known as the NADPH oxidase. Two of the essential cytosolic components of the NADPH oxidase are p47-*phox* and p67-*phox*. Currently, only the human and murine homologues of these proteins have been sequenced. Because of the important role neutrophils play in bovine host defense, I carried out studies to clone, sequence, and express bovine p47-*phox* and p67-*phox*. Using PCR cloning techniques and a bovine bone marrow cDNA library, I cloned both of these bovine NADPH oxidase cytosolic components. Comparison of the bovine sequences with those of the human and murine homologues showed that they were highly conserved, but also revealed important information regarding key structural features of p47-*phox* and p67-*phox*, including location of putative phosphorylation sites. Functional expression of bovine p47-*phox* and p67-*phox* showed that these proteins could substitute for the human proteins in reconstituting NADPH oxidase activity in a cell-free assay system, again demonstrating the high degree of conservation between human and bovine homologues. This study greatly contributes to our understanding of the potential structural/functional regions of p47-*phox* and p67-*phox* as well as gives us information that can be used to study the role of neutrophils in bovine inflammatory diseases.

INTRODUCTION

Neutrophils play an essential role in host defense against bacterial and fungal pathogens (1). These inflammatory cells also play an important role (2) in serious inflammatory diseases, such as arthritis and adult respiratory distress syndrome (3,4). A key component in host defense is the NADPH oxidase system, which becomes activated when neutrophils contact and ingest foreign pathogens. The activated NADPH oxidase generates large quantities of superoxide anion (O_2^-) through reduction of molecular oxygen by NADPH-derived electrons (5). In addition, O_2^- is subsequently converted to other microbicidal products, such as H_2O_2 and HOCl. The NADPH oxidase has been most extensively studied in human neutrophils; however, the mammalian species investigated to date have also been shown to produce proteins corresponding to the membrane-bound proteins gp91-*phox* and p22-*phox* (6-10) and the cytosolic proteins p47-*phox* (10-16), p67-*phox* (10,14,16-18) and p40-*phox* (18,19). While conservation of these essential proteins would be expected, they have been characterized only in a few species (6-9,11-19).

Importance of Neutrophils in Host Defense

Across species, neutrophils play an important role in host defense, and a number of disease states have been associated with defective neutrophil function. In cattle, as in humans, these diseases are characterized by enhanced susceptibility to infections. For example, a genetic defect in CD18 (the common β-subunit of all β_2 integrins) is the cause of bovine leukocyte adhesion deficiency (BLAD) and its human counterpart, leukocyte

adhesion deficiency (LAD) (20). This defect severely impairs leukocyte adhesive functions, and neutrophils from animals with BLAD or humans with LAD fail to adhere to the vascular wall and therefore, cannot migrate into the tissue (20). An analog of human Chediak-Higashi syndrome, characterized by abnormal pigmentation, atypical azurophilic granules, and impaired bactericidal activity has also been described in cattle (21,22). Neutrophils also play an essential role in the cellular defense of the bovine mammary gland, where compromised leukocyte function has been linked to the development of bovine mastitis, the most common and costly disease afflicting dairy cattle around the world (23). The effectiveness of this defense system depends on the promptness and the magnitude of the neutrophil migratory response, as well as the phagocytic and bactericidal activities of these cells (24).

In cattle, neutrophil-derived products have been associated with adverse effects in acute pulmonary diseases, such as pulmonary thromboembolis, endotoxic shock, acute respiratory distress syndrome, and immune complex interstitial pneumonia. The pathogenesis of neutrophil-mediated injury has been postulated to be secretion and/or release of the toxic reactive oxygen species the neutrophil uses in microbial defense. As early as 1985, Slocombe *et al.* found neutrophils to be required for initiation of acute lung injury in neonatal calves with experimentally induced pneumonic pasteurellosis (25,26). The presence of high numbers of activated leukocytes in mammary tissues, which occurs in mastitis, also contributes to inflammatory tissue damage, including fibrosis, swelling and atrophy of the mammary tissue (24).

Hematopoiesis – Stem Cell to Mature Granulocyte

While neutrophils comprise about 60-75% of the blood leukocytes in most mammals, the blood of cattle contains only 20-30% neutrophils (27,28). Neutrophils originate in the bone marrow of all bones at birth, but only the flat bones continue hematopoiesis throughout life. Neutrophils begin as a blast-type cell, the myeloblast, and progress through maturation stages designated as progranulocyte, myelocyte, metamyelocyte, band and segmented neutrophil. In the band stage, the smooth nuclear membrane begins to indent at several points as the cells mature. At this stage the neutrophils are released into the peripheral blood where they age and the nucleus becomes segmented into several distinct lobes separated by filaments. Maturation of neutrophils from precursor cells requires approximately 5 to 6 days (29). The cells only remain in the blood for a few hours before entering the tissue. When neutrophils leave the blood to enter the tissues, a bone marrow reserve is stimulated to replenish those cells (27).

Electron microscopic analysis of bovine neutrophils shows them to be relatively small (diameter of about 7-10 μm), rounded or slightly elongated cells with few cytoplasmic projections (30,31). The nucleus of a neutrophil is distinct from other nucleated eukaryotic cells due to its size, shape, and a low number of nuclear pores (31). It is primarily composed of heterochromatin and seldom contains a nucleolus (31). The presence of a small drumstick-shaped lobe known as the female sex chromatin lobe is only an occasional occurrence in the bovine neutrophil (27). Several spherical, elongated, rod and dumb-bell shaped granules can been seen in the dense cytoplasm. A roughly circular, granule-free area

near the nuclear lobes contains stacks of four to nine Golgi cisternae arranged around one or two centrioles. The cytoplasm also contains particulate glycogen and a few vesicular profiles of rough-surfaced endoplasmic reticulum, but lacks appreciable quantities of most other organelles (e.g., mitochondria, ribosomes, and microtubules) (31). A narrow rim of cytoplasm around the periphery of the neutrophil is generally free of organelles and inclusions, but is rich in cytoskeletal proteins (31).

Unlike the nearly invisible granules of neutrophils from other domestic animals, the granules of bovine neutrophils are faintly visible in the cytoplasm of the cell (31). Three types of granules are present in bovine neutrophils; each type of granule arising at a different stage of cell maturation (32). The level at which the genes for granule components are sequentially activated is unknown. It is likely that their biosynthesis shutdown, which is coupled to the progress of myeloid differentiation, is carried out at the level of transcription (33). Peroxidase-positive granules, analogous to azurophil or primary granules from other species, are present in relatively small numbers. Primary granules are round or elongated and contain microbicidal enzymes (myeloperoxidase, acid hydrolases, and neutral proteases). These enzymes function in concert with membrane-bound and cytoplasmic enzymes to kill and digest phagocytized microbes, but can also be responsible for inducing tissue damage at sites of inflammation. The azurophilic granules also contain cationic proteins and other non-lysosomal enzymes (31). Compared with human neutrophils, bovine neutrophils lack lysozyme and show decreased levels of the azurophilic granule enzymes, such as β-glucuronidase, β-galactose, and myeloperoxidase. A survey of bovine granulocytes has indicated that myeloperoxidase is particularly concentrated in bovine eosinophils, suggesting

that the level of myeloperoxidase activity in some preparations of bovine neutrophils might reflect, in part, the presence of contaminating eosinophils (30,34,35).

The two remaining granule types, the large and specific granules, are round, peroxidase-negative, and distinguishable by size. The large granules are greater than 0.35 μm in diameter, have a pale, very homogenous matrix. These granules are found in the cytoplasm of bovine neutrophils, but not in neutrophils from other animals. Analysis of bone marrow specimens has shown that the formation of the large granules occurs in the myelocyte stage after the production of the peroxidase-containing azurophilic granules (32,33). A number of highly cationic proteins that are not found in other subcellular compartments are localized in the large granules. These proteins are thought to be responsible for the non-oxidative antimicrobial activity associated with this granule type. Unlike the azurophil or specific granules, the large granules contain no serine proteases or metalloproteases, acid hydrolases, or peroxidase (33,36).

The smaller, specific granules have a diameter of less than 0.3 μm and are formed after most of the large granules have matured. In other species, specific granules have been shown to contain lactoferrin and approximately 66% of the neutrophil's lysozyme (31). The concentrations of lactoferrin and vitamin B_{12}-binding protein, markers of specific granules in other species, appear to be higher in bovine cells than in the human neutrophils. However, as in the bovine azurophilic granules, lysozyme activity is undetectable in specific granules of this species (30). Bovine specific granules do have strong, nonspecific alkaline phosphatase activity, but show no acid phosphatase activity (31). Table 1.1 summarizes the major granule components that are known to exist in bovine neutrophils.

Table 1.1. Content and Function of Bovine Neutrophil Granules

Type of Granule	Factor	Function
Azurophilic (Primary)	Myeloperoxidase	Catalyzes the production of hypohalus acids for microbicidal activity
	Acid Hydrolases Arylsulfatase, β-glucuronidase β-galactosidase	Degrades some microbial macromolecules; degrades glucuronic acid and galactose in some bacterial capsules
	Neutral acid proteases Elastase Serine proteases Cathespin G	Breakdown of extra-cellular connetive tissue components; produce kinin-like mediators; alter vascular permeability; digest collagen, cartilage, and elastin; activate proforms of cationic proteins found in large granules. Kills Gram-positive and some Gram-negative microorganisms
	Acid phosphatase	Monophosphate esterase
Specific (Secondary)	Lactoferrin	Chelates iron. Binds to negatively charged cell surfaces and may deprive bacteria of iron. Catalyst causing O_2^- and H_2O_2 to generate $OH^•$; functions with antibody and possibly lysozyme for antimicrobial activity; contributes to neutrophil migration, increases adherence and aggregation.
	Collagenase	Digests microbial macromolecules
	Vitamin B_{12}-binding Protein	Binding and transport of vitamin-B_{12}
	Alkaline phosphatase	Monophosphatase esterase
Large	Cationic Proteins Bactenecins Prododecapeptide ProBMAP28	Antimicrobial peptides; phospholipase and chymotrypsin-like activity; Decrease cell surface charges; Facilitates neutrophil adherence and aggregation
	Lactoferrin	Chelates iron. Binds to negatively charged cell surfaces and may deprive bacteria of iron. Catalyst causing O_2^- and H_2O_2 to generate $OH^•$; functions with antibody and possibly lysozyme for antimicrobial activity; contributes to neutrophil migration, increases adherence and aggregation.

Table adapted from references 30, 34, 36, 90.

Neutrophil Response to Pathogenic Stimuli

Neutrophils capture and destroy dying cells, microorganisms, or foreign material by means of phagocytosis. Although a continuous process, phagocytosis can be divided into the distinct stages of adherence, chemotaxis, ingestion and digestion (28).

Adherence

Recruitment of neutrophils to the site of acute inflammation involves the combined action of multiple families of adhesion molecules, cytokines, and chemoattractants. The extravasation of neutrophils from the bloodstream initially depends on adhesive interactions. These adhesive interations are mediated through the selectins, which bind sialylated and fucosylated oligosaccharide ligands, such as sialyl Lewis X (sLe^x) (37-39). L-selectin, a protein expressed the neutrophil surface, binds to constitutively expressed ligands on high endothelial venules of peripheral lymph nodes [e.g., mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and peripheral lymph node addressin (PNAd)], to undefined ligands upregulated on endothelial cells at sites of inflammation, and to inducible ligands on other leukocytes [e.g., P-selectin glycoprotein ligand-1 (PSGL-1)] (38). On the endothelial cells, E-selectin and P-selectin are upregulated to bind to carbohydrate ligands on the surface of the neutrophil, including E-selectin ligand 1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1) (37-39). The selectin-mediated interactions facilitate leukocyte rolling, slowing the flow of neutrophils through venules, and allow time for the neutrophils to sample the local environment as well as the endothelial cell surface. Binding of the selectins to their

ligands and the presence of chemoattractants can enhance the expression and adhesiveness of another receptor on the neutrophil surface, Mac-1 (CD11b/CD18). Mac-1, a member of the β_2-integrin family of leukocyte adhesion molecules, allows neutrophils to bind tightly to activated endothelium through its ligand, intercellular adhesion molecule-1 (ICAM-1) (40,41). In response to acute inflammatory stimuli, such as (42,43)interleukin-1 (IL-1), interleukin-8 (IL-8), platelet activating factor (PAF), or lipopolysaccharide (LPS), the neutrophil surface expression of Mac-1 is upregulated, while L-selectin is down-regulated due to cleavage by a specific protease (44,45). Neutrophils bound to ICAM-1 remain attached to the vessel wall and are, therefore, able to migrate along the endothelial surface and through the vessel wall into tissues along a concentration gradient of chemoattractant (43,46,47).

Chemotaxis

Bacterial invasion and the ensuing inflammatory response often results in the production of many different chemotactic molecules. As chemotactic molecules diffuse from the site of tissue damage, a concentration gradient is formed in the tissue and can be detected by adherent neutrophils. Bovine neutrophils have been shown to respond to opsonized particles or bacteria (48-50) as well as inflammatory mediators including prostaglandins (51), leukotriene B_4 (52,53), platelet activating factor (43,49,54), complement fragment C5a (55,56) and cytokines such as IL-1 (51), IL-8 (56,57), tumor necrosis factor (TNF-α) (58), and granulocyte-colony stimulating factor (59). An interesting characteristic of bovine neutrophils is the lack of a chemotactic response to the bacterial N-formylated peptides such

as fMLF (60,61). Formylated peptides have been shown to stimulate chemotaxis of primate, rat, rabbit, and guinea pig neutrophils, while like bovine cells, this response is not seen in porcine, feline, and canine neutrophils [reviewed in (53)].

Although the stimulus may vary, the mechanics of neutrophil movement appear to be conserved among species. Neutrophils migrate toward a chemotactic source with a broad leading edge (lamellipodium), which contains the actin network, and a knob-like posterior (uropod) (62,63). Actin, a major cytoskeletal protein, is responsible for the cytoskeletal changes that correspond with cell movement (49,64). In response to a chemotactic signal, monomeric actin assembles into filaments and microtubules. Microtubules extend to, but do not penetrate the lamellipodium and uropod. Microfilaments form a zone subjacent to the plasma membrane excluding cell organelles, where electron-dense polymerized areas serve as sites of cell attachment (65). The nucleus moves to the rear of the neutrophil and is separated from the lamellipodia by most of the granules and the Golgi apparatus (62,65). In polarized cells, the uropod membrane contains almost all of the coated pits and vesicles, as compared to the diffuse distribution found in unstimulated neutrophils. Before the neutrophil can change its direction of migration, reorientation of the nucleus, organelles and cytoskeletal elements must occur (62). Neutrophils are extremely sensitive to variations in chemoattractant concentration, and human neutrophils can detect as little as a 0.1% gradient difference between different regions of the cell surface (66). Changes in the diffusion time and the orientation of the stimulant may determine whether directional locomotion results in neutrophil aggregation (55), enhanced oxidative metabolism (51), or augmentation of complement receptor expression (67). These activities in turn modulate the chemotactic

response. Newly generated active oxygen radicals initiate the production of chemotactic and chemokinetic factors from cellular and extracellular lipids that may modify neutrophil function (51). When macrophages encounter invading organisms, they release IL-1 and TNF-α, which recruit neutrophils into inflamed tissues and prime these cells for a heightened oxidative burst to assist in microbial killing (58).

The response of neutrophils to a given chemoattractant is usually transient and results in a decreased responsiveness to subsequent stimulation by the same agonist (68). This type of homologous desensitization has been proposed to result from receptor phosphorylation, receptor association with the cytoskeleton, and receptor internalization (68,69). Heterologous desensitization can also occur and is characterized by the loss of responsiveness by a given receptor (e.g., C5a receptor or IL-8 receptor) following activation of a different receptor or process (e.g., fMLF receptor) (68,70). However, desensitization is highly dependent on the order in which the cell encounters the chemoattractant, and treatment of the neutrophils with IL-8 has no effect on the subsequent response to fMLF (70). The ability of chemoattractant receptors to "talk" with each other in this manner may help regulate leukocyte migration in the presence of complex chemoattractant arrays which are found at sites of inflammation (70).

Ingestion

When a neutrophil encounters a foreign particle, it extends pseudopodia around it, and binding occurs between opsonins on the organism and receptors on the neutrophil. Once bound firmly to the neutrophil surface, the particle is drawn into the cell and becomes

enclosed in a vacuole called a phagosome [Reviewed in ref. (28,31)].

Ingestion and degranulation are interrelated processes, which require intact neutrophil membranes and cytoskeletal elements (microbutules and microfilaments). When a phagocytizable particle binds to the cell membrane, cytoskeletal changes occur which are similar to those seen with chemotaxis. Neutrophil pseudopodia flow around the particle by the coordinated action of microfilament polymerization, changes in cell membrane fluidity, and receptor binding (31). The ease of this enclosure depends, in part, on the surface of the particle. Neutrophil cytoplasm readily flows over hydrophobic surfaces so that hydrophobic bacteria, such as *Mycobacterium tuberculosis,* are rapidly ingested. In contrast, *Streptococcus pneumoniae* has a hydrophilic carbohydrate capsule and is poorly phagocytosed unless made hydrophobic by a coating of antibodies or C3b (28). While opsoninization of a particle increases the rate of phagocytosis several fold, it is not required (34). In addition, the rate of phagocytosis is different between species of domestic animals. For example, bovine peripheral blood neutrophils ingest opsonized *Staphylococcus aureus* more rapidly than equine neutrophils (31).

The membranes near pseudopodia accumulate ligand-receptor complexes and are enriched in "coated" membranes. Endocytotic function is restricted to this region. The lamellipodial membrane excludes coated pits and lacks pinocytotic activity, but has preferential binding of immunoglobulin aggregates (Fc receptors). The uropodia of a neutrophil undergoing chemotaxis are characterized by receptor-mediated endocytosis, pinocytosis, and a high concentration of coated pits and vesicles. In neutrophils, the CD32 receptor, known as FcRγII, binds the Fc region of antibody molecules, triggers the respiratory

burst, and initiates ingestion. Another receptor, CD35 (or CR1) binds the complement component, C3b. Binding of C3b-coated particles leads to attachment, but may not necessarily trigger ingestion (31,51).

Destruction

Once a pathogen is phagocytosed, cytoplasmic granules fuse with the phagocytic vacuole and discharge their contents into the phagosome as a mixture of enzymes, including myeloperoxidase and highly reactive oxidizing agents associated with the respiratory burst (71). The antimicrobial systems of neutrophils can be classified into oxygen-dependent and oxygen-independent systems. The oxygen-dependent system involves consumption of molecular oxygen and production of O_2^-, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hyroxide radical ($OH^\bullet$), and singlet oxygen ($^1O_2$). The oxygen-independent system is more diverse and is composed of various factors, including acid pH, lysozyme, cationic proteins, lactoferrin, vitamin B_{12}-binding protein, and acid hydrolases. The combination of various factors involved with killing a given type of microbe or in causing tissue damage is not well defined, and it is likely that all of these components participate to some in both of these processes (31).

The earliest events of degranulation are associated with changes in granule morphology. Degranulation, like phagocytosis, depends on an intact cytoskeletal system and is closely linked to chemotaxis, ingestion, and neutrophil metabolism. Secretion of granule proteins, such as elastase and collagenase at the lamellipodial edge, helps loosen the interstitial matrix promoting neutrophil migration (51). Granule enzymes may be autocrine-

like, controlling neutrophil function by destroying the hyaluronic acid, which can modulate neutrophil adhesion and directed migration. Granule exocytosis also releases mediators that influence the inflammatory response by inactivating as well as generating chemoattractants (31,51).

Microtubule and microfilament polymerization are required to move granules to the phagosomal membrane for intracellular release or to the plasma membrane for extracellular release. Release of granule enzymes initiates neutrophil oxidative metabolites, leading to microbial killing, tissue damage, and modulation of various inflammatory and immunologic processes.

Microbicidal Mechanisms of Bovine Neutrophils

Oxygen-independent killing

The oxygen-independent system is composed of numerous components, including cationic proteins, lactoferrin, vitamin B_{12}-binding protein, and acid hydrolases. Note, however, that oxygen-independent mechanisms are not strictly dependent on anaerobic conditions, as many of these mechanisms can function in the presence or absence of oxygen.

After phagocytosis, the hydrogen ion (H^+) concentration within the phagosome increases rapidly, likely as a result of the lactate produced by the glycolysis that occurs during phagocytosis. The low pH level in the phagosome may be microbicidal (attacking organic acids and lipophilic acids) or microbiostatic, depending on the sensitivity of the microbe to changes in pH. Acid pH also facilitates the function of some microbicidal enzyme systems (myeloperoxidase-H_2O_2-halide system, acid hydrolases, and lysozyme).

Enzymes considered to have a digestive role (hydrolases) function best at an acid pH; however, cationic proteins have a pH optimum at seven or above (72,73).

Lysozyme, a cationic protein that attacks β1,4-glycosidic bonds in bacterial cell walls, is found in both the azurophilic and secondary granules of some animal neutrophils, but requires other antimicrobial factors to induce microbial killing (72). Compared to human neutrophils (~30 μg), the amount of lysozyme in bovine neutrophils (~0.2 μg) is small, so the contribution of this enzyme to the overall killing and digestive functions is probably not significant (31,74).

Lactoferrin, from specific granules, inhibits bacterial growth by binding iron; however, a reduced ability to kill phagocytized *Escherichia coli* by lactoferrin-deficient neutrophils suggests a role in the killing of some microbes (31). One possibility is that lactoferrin, antibody, and lysozyme may function in concert to exert antimicrobial activity, although this has not been conclusively demonstrated. Lactoferrin also increases granulocyte adherence and aggregation and reduces the cell surface charge, contributing to the control of neutrophil migration and oxygen-radical production (31). Interestingly, bovine neutrophils have a higher concentration of lactoferrin than that of human granulocytes (74), suggesting lactoferrin may play a more important role in bovine neutrophil function.

Azurophil granules also contain cationic proteins, such as neutral and acid proteases that are associated with the breakdown of extracellular connective tissue components. These proteases are involved in the production of kinin-like mediators (leukokinins from serum proteins), the alteration of vascular permeability (possibly mediated by leukokinin), and the digestion of various types of collagen, cartilage, and elastin (75). Enzymatic activity is not

necessary for antimicrobial activity by these proteins. Cationic proteins bind to the microbe and impair replicative ability without altering the bacterial structure. These proteins likely act in concert with other antimicrobial mechanisms and may enhance the killing effects of the myeloperoxidase-H_2O_2-halide system (73).

Two peptide families have been isolated from neutrophil granules: the defensins and bactenicins. Defensins have been found in human, rabbit, rat and guinea pig neutrophils. Neutrophils from cattle and other ruminants also contain potent antimicrobial peptides; however, they are distinct from other species and are known as bactenecins. Bactenecins constitute an efficient cell-dependent defense system against bacteria, viruses, and parasites (33). These cyclic, defensin-like peptides are found in the large granules of bovine neutrophils. Bactenicins are highly cationic polypeptides with molecular weights ranging from 1,600 to 8,000 daltons (33). These peptides exert activity in a wide range of pH conditions (maximum pH 7-8) and remain active in physiological solutions (74). Two of these peptides, Bac5 and Bac7, have been shown to contain high levels of arginine and proline (> 60%), and have unique primary structures with repeated sequences. The Bac7 structure contains an "arginine-clustered region" (8 arginine residues within residues 1-17) and "three tandem repeats" of a tetradecapeptide (residues 18-31, 32-45, and 46-59). It has been suggested that Bac7 exhibits antimicrobial activity due to a bacteriostatic rather that a bacteriolytic effect (76). The peptide's mechanism of action involves binding to the outer membrane of Gram-negative bacteria, followed by a rapid translocation to the inner membrane, where the electron transport chain and some energy dependent membrane activities are impaired (33). Bactenicins appear to be stored in the large granules as harmless

propeptides, requiring cleavage by neutral serine protease(s) to generate antimicrobial molecules. In stimulated neutrophils, probactenecins are activated after their discharge into the phagocytic vacuoles by neutral serine proteases, such as elastase or cathepsin G, that are released into the phagosome by azurophil granules (33,74).

Oxygen-dependent killing by bovine neutrophils

Within seconds of binding to a foreign particle or stimulation of an antibody receptor, such as CD32, neutrophils increase their oxygen consumption nearly 100-fold. This increase in respiration results from activation of the respiratory burst oxidase or NADPH oxidase (27,77). The NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen, converting it into superoxide anion (O_2^-), which is released into the phagosome or outside of the plasma membrane [Reviewed in ref. (78)] (See Reaction 1).

$$NADPH + 2O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} NADP^+ + H^+ + 2O_2^- \qquad \text{Reaction 1}$$

O_2^- is intrinsically unstable and spontaneously reacts to form secondary metabolites such as H_2O_2, HOCl, $OH^\bullet$, $^1O_2$, and $ONOO^-$ (79-84). O_2^- can also be scavenged by the enzyme superoxide dismutase (SOD) which is found in most tissues. SOD dismutates two molecules of O_2^- to generate one molecule of H_2O_2 (See Reaction 2).

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \text{Reaction 2}$$

As H_2O_2 accumulates in the cell, it can be scavenged by catalase which converts H_2O_2 intoH_2O (See Reaction 3). H_2O_2 can also be converted to other bactericidal compounds

through the action of myeloperoxidase from the primary granules (75). Myeloperoxidase (MPO) catalyzes a reaction between H_2O_2 intracellular halide ions (Cl^-, Br^-, I^-, or SCN^-) to produce hypohalides (See Reaction 3).

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} H_2O + HOCl \qquad \text{Reaction 3}$$

The chloride ion is probably used at most sites *in vivo* except in milk and saliva where SCN^- is employed (27). Hypochlorite ion (OCl^-) is a major product of neutrophil oxidative metabolism and kills bacteria by oxidizing bacterial proteins and enhancing the bactericidal activities of the lysosomal enzymes (27).

The reduction of H_2O_2 produces $OH^{\bullet}$, which is highly unstable and reacts with lipids and nucleic acids. It initiates lipid peroxidation by removing hydrogen atoms from unsaturated lipids to form hydroperoxides, which cause severe damage to cell membranes, organelles and associated enzymes (81).

Singlet oxygen is formed when oxygen absorbs sufficient energy to shift one of its unpaired electrons from its lowest energy level, or ground state, into an orbital of higher energy. The distorted electron shell of singlet oxygen has an affinity for compounds containing double bonds. Thus, polyunsaturated fatty acids can be attacked, resulting in the formation of hydroperoxides (71).

The reaction of O_2^- with nitric oxide, which is produced by neutrophils themselves as well as vascular endothelial cells, produces a toxic oxidant, peroxynitrite ($ONOO^-$) that is able to attack a wide variety of tissues (83). Recent research has implicated $ONOO^-$ as one of the damaging agents in a number of inflammatory diseases in humans (84). The formation

of $ONOO^-$ has also been shown to prime human neutrophils at sites of inflammation, increasing the efficiency by which neutrophils kill microorganisms (84).

Since all of these oxygen radical species are generated intracellularly, it is inevitable that some of these products leak into the cytoplasm. Thus, the cytoplasm is protected against the oxidative effects by the scavenging enzymes, SOD and catalase. H_2O_2 can also be removed by coupling to the glutathione reductase system, generating $NADP^+$ and subsequently increasing hexose monophosphate (HMP) shunt activity (71). Bovine neutrophils have low catalase activity, but high levels of two enzymes of the glutathione pathway, glutathione peroxidase and glutathione reductase, which may be the method used more often in this species (30). Defense mechanisms of bovine neutrophils are summarized in Figure 1.1.

NADPH Oxidase Components in Bovine Neutrophils

In the resting cell, the O_2^- generating machinery is dormant, and its protein components are segregated into cytoplasmic and plasma membrane compartments. Activation of the NADPH oxidase involves the interaction and assembly of several neutrophil proteins, and the transfer of electrons from NADPH to molecular oxygen occurs only after the cytosolic components of the oxidase are translocated to and assembled with the membrane-bound components. The membrane and cytosolic proteins involved in NADPH oxidase assembly are listed in Table 1.2.

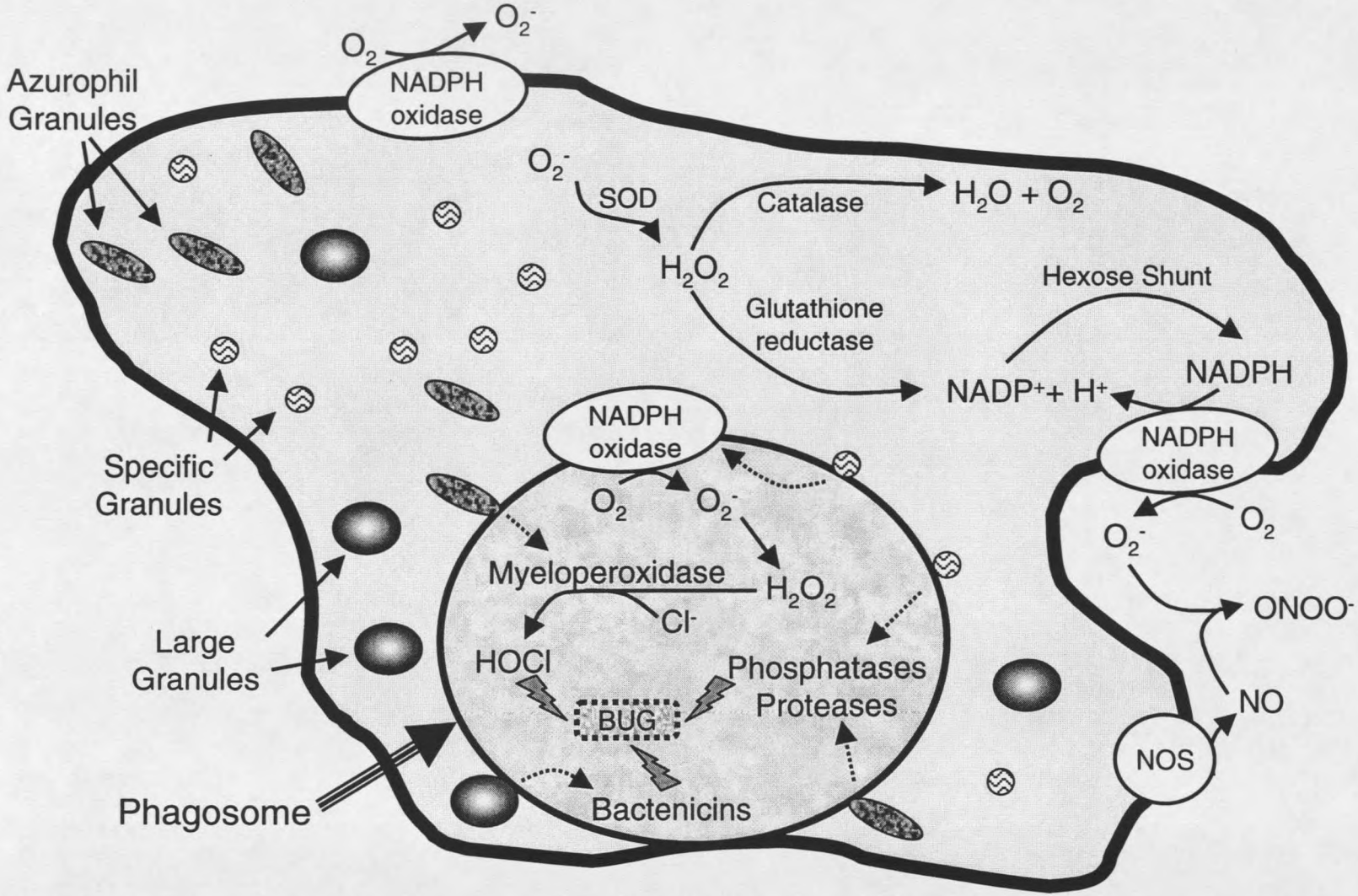

Figure 1.1. Schematic Representation of Bovine Neutrophil Microbicidal Mechanisms

Table 1.2. NADPH Oxidase Proteins

Membrane Components	Cytosolic Components
Flavocytochrome b	p40-*phox*
gp91-*phox*	p47-*phox*
p22-*phox*	p67-*phox*
Rap1A	Rac

Membrane Components

Flavocytochrome b is an integral membrane protein (5,6,85) composed of two subunits, gp91-*phox* and p22-*phox*, which are present at a 1:1 molar ratio (86). Studies have shown that both subunits are required for functional surface expression of the flavocytochrome b assembly, and a defect in one of the subunit proteins results in decreased expression of the other subunit (87). The heterodimer is responsible for coordinating at least two hemes, one which may be shared between gp91-*phox* and p22-*phox*. The larger subunit, gp91-*phox*, has 4-5 transmembrane helices (78), five potential glycosylation sites in the amino-terminal region, and has been shown to contain FAD and NADPH binding activities (88,89). The small subunit, p22-*phox*, has a molecular weight of 22 kDa and contains hydrophobic helices in the N-terminal portion of the protein that could also serve as membrane-spanning domains (87). Flavocytochrome b appears to act as the focal point of NADPH oxidase assembly, and the translocation of the cytosolic components does not occur in the absence of flavocytochrome b (90,91). In addition to the flavocytochrome b heterodimer, a low molecular weight GTP-binding protein, Rap1A, is also membrane bound

and associates with flavocytochrome b (92,93). In humans, Rap1A appears to play a regulatory role in the NADPH oxidase (78,94). A Rap homologue exists in cattle (95) and may function similar to human Rap1A, but this has yet to be determined.

Bovine gp91-*phox* has been sequenced by our lab and was shown to encode a protein of 570 amino acids. In addition to being identical in length to human and murine gp91-*phox*, the bovine protein is ~92% identical to the other species sequenced to date. Five potential glycosylation sites are located in regions similar to human gp91-*phox*, further suggesting that this protein is highly conserved across species. Bovine p22-*phox* has also been sequenced by our lab and was shown to be ~86% identical to homologues from other species (9).

Cytosolic components

Since the active enzyme complex forms at the membrane, the cytosolic proteins must translocate from the cytosol to associate with membrane-bound flavocytochrome b and possibly the cytoskeleton (Figure 1.2.). The human cytosolic NADPH oxidase proteins include p40-*phox*, p47-*phox*, p67-*phox* and a second low molecular weight GTP-binding protein, Rac2 [Reviewed in refs. (78,96,97)]. Extensive research of these proteins has been carried out in human and murine neutrophils. The isolation of bovine cytosolic proteins was not reported until 1990 (98). Since the functional role of the NADPH oxidase appears to be conserved across species, conservation of the cytosolic proteins would be expected. However, differences have been found to occur between species. For example, the importance of p47-*phox* and p67-*phox* has been demonstrated by the recurrent infections that

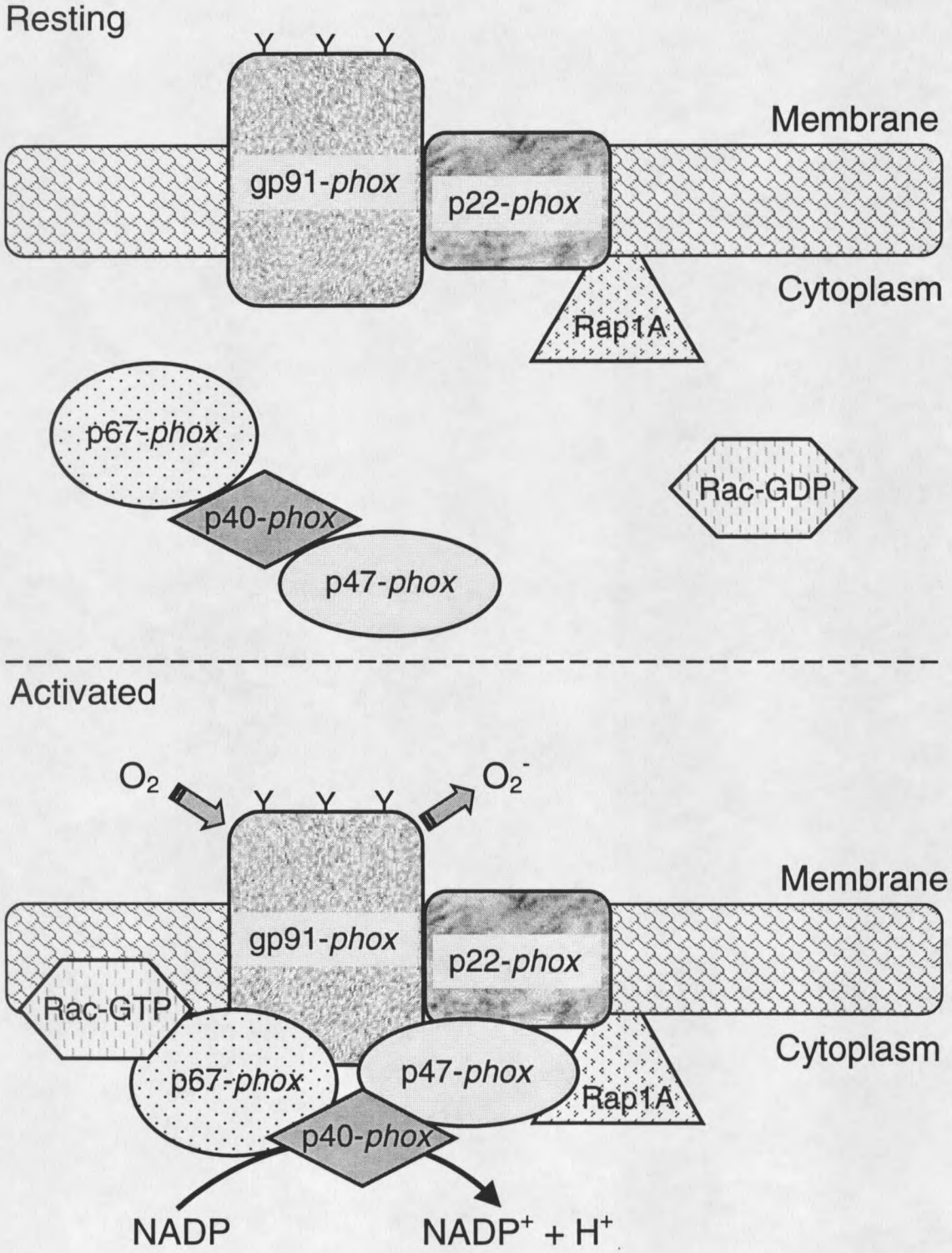

Figure 1.2. Hypothetical Model of the NADPH Oxidase Assembly Process

occur in individuals with chronic granulomatous disease (CGD), a disease often caused by defects in these proteins (91,99). However, a CGD counterpart has not been found to occur in cattle or mice.

In the cytosol of resting human cells, p40-*phox*, p47-*phox* and p67-*phox* are proposed to exist in equimolar concentrations in a 240 kDa complex. Upon activation, this complex translocates to and associates with the plasma membrane bound components. Free p47-*phox* is also found in the cytosol and can be transferred to the membrane (97,100,101). The assembled complex of flavocytochrome b, p47-*phox*, and p67-*phox* has been reported to be equimolar (102), with an apparent irreversible complex stabilized by association between p47-*phox* and flavocytochrome b (103). Rac also translocates to the plasma membrane, independent of the 240 kDa cytosolic aggregate, and associates with the membrane-bound components (104).

In human cells, p47-*phox* appears to be the first cytosolic component to interact with flavocytochrome b (90,97,102), and its translocation to and association with flavocytochrome b is a prerequisite for p67-*phox* translocation and subsequent production of O_2^- (90,97,102,105). Only about 5-10% of the p47-*phox* is translocated to the membrane in human cells and only a fraction of the transferred p47-*phox* is involved in O_2^- production. Both human and murine p47-*phox* have been shown to undergo extensive phosphorylation and have been suggested to be the activation signal for NADPH oxidase (97,101,106-109). The association of p47-*phox* with other NADPH oxidase components may be modulated by Src homology 3 (SH3) domain interactions (13,110-113). Human p67-*phox* SH3 domains have been reported to play a role in the formation of the 240 kDa complex in resting

neutrophils and to assist in assembly of the activated oxidase (18,113-125).

P67-*phox* is proposed to be the limiting factor in neutrophil cytosol (126), and it has been reported that three-fold less p67*phox* than p47-*phox* is present in human neutrophils (101). This may indicate that most or all of the p67-*phox* is associated with p47-*phox* or resides within the 240 kDa complex. Most reports agree that the translocation and membrane association of p67-*phox* is dependent on the co-translocation of p47-*phox* to the plasma membrane and prior interaction of p47-*phox* with flavocytochrome b (90,102,103,123,127).

As with p47-*phox* and p67-*phox*, human p40-*phox* resides within the 240 kDa complex in neutrophil cytosol. Its function is unknown, but p40-*phox* has been reported to associate with p67-*phox* through a non-SH3 related interaction (78,120,128) and it may function to stabilize p67-*phox* (128). Following neutrophil activation, p40-*phox* has been reported to co-translocate as part of the 240 kD complex from the cytosol to the plasma membrane. Human p40-*phox* contains a single SH3 domain and shows significant homology with the N-terminus of p47-*phox*.

Rac proteins function as molecular switches, cycling between GDP in their inactive form to acquiring and hydrolyzing GTP in the active state (129). During neutrophil activation, Rac has been reported to translocate in equimolar amounts (104) to the membrane independent of the other cytosolic factors (130). The first 199 amino acids of p67-*phox* have been shown to bind Rac *in vitro* (131). Rac apparently does not regulate assembly, but may regulate the activated oxidase complex (127,132).

Project Goals

The goal of this research project is to increase the knowledge of the bovine NADPH oxidase system by characterizing two cytosolic NADPH oxidase proteins, p47-*phox* and p67-*phox*. Chapter 2 describes the cloning and sequencing of bovine p47-*phox* and p67-*phox* proteins and compares the bovine sequences to the homologous nucleotide and protein sequences found in human, mouse, and rabbit neutrophils. The similarity of the sequences between these species is striking, indicating that the neutrophil NADPH oxidase system has been highly conserved throughout mammalian evolution. These proteins were also expressed in a mammalian cell line to investigate functional similarity by testing the ability of the bovine cytosolic proteins to activate the membrane components from human neutrophils. A better understanding of the bovine NADPH oxidase components will enable further study of the neutrophil's role in bovine inflammatory disease, as well as strengthen out knowledge of this important system across species.

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CLONING AND EXPRESSION OF BOVINE P47-*PHOX* AND P67-*PHOX*: COMPARISON WITH THE HUMAN AND MURINE HOMOLOGUES

Introduction

Polymorphonuclear leukocytes or neutrophils play an important role in the body's host defense against bacterial and fungal pathogens (1,2). Neutrophils respond to the presence of a pathogen by migrating to its location, phagocytosing the pathogen, and generating microbicidal oxidants designed to kill the pathogen (1,2). The generation of microbicidal oxidants by neutrophils results from the activation of a multi-protein enzyme complex known as the NADPH oxidase (3,4). The NADPH oxidase is responsible for transferring electrons from NADPH to O_2, resulting in the formation of superoxide anion (O_2^-) (3,5). O_2^- is rapidly converted to secondary toxic oxygen species, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and hypochlorous acid (HOCl), that can efficiently kill microorganisms and, in combination with the primary and secondary granule contents, comprise the primary host defense mechanism used by neutrophils (6). The importance of the NADPH oxidase to host immunity is demonstrated by the recurrent infections that occur in individuals with chronic granulomatous disease (CGD), a disease resulting in genetic defects of the NADPH oxidase system (7,8).

Activation of the NADPH oxidase system involves the interaction and assembly of several neutrophil proteins, some located in the plasma membrane, and others in the cytosol [reviewed in reference (9)]. The most important membrane-associated protein is flavocytochrome b_{558} (known as flavocytochrome b), which functions as the redox center of

the oxidase and is a heterodimer of two tightly associated polypeptides (gp91-*phox* and p22-*phox*) (10-12). Also in the membrane is a low molecular weight GTP-binding protein (LMWG), Rap1A, that associates with flavocytochrome b (13) and plays a role in regulating the NADPH oxidase *in vivo* (14). The cytosolic NADPH oxidase proteins include p40-*phox*, p47-*phox*, p67-*phox*, and a second LMWG, Rac2 [reviewed in references (3,4,9)]. Upon neutrophil stimulation, the cytosolic proteins translocate to the membrane and associate with flavocytochrome b and possibly the cytoskeletal system [reviewed in references (3,4,9)].

Through analysis of neutrophils from patients with CGD, both p47-*phox* and p67-*phox* have been shown to be absolutely required for NADPH oxidase activity (7,8). However, the mechanism by which these cytosolic proteins assemble with flavocytochrome b during activation of the NADPH oxidase is complex and not completely understood. Previous studies by several groups have demonstrated that p47-*phox* and p67-*phox* exist in a complex in the cytosol (15,16); however, p47-*phox* seems to be the first cytosolic component to interact with flavocytochrome b during the assembly process (17,18), and its association with flavocytochrome b is a prerequisite for binding of p67-*phox* (17-19). During NADPH oxidase activation, p47-*phox* has been shown to undergo extensive phosphorylation (20-23), and agents that stimulate phosphorylation of p47-*phox* in intact neutrophils (such as phorbol esters) can also stimulate O_2^- production (24). Furthermore, inhibition of phosphorylation during neutrophil activation by the protein kinase C (PKC) inhibitor staurosporine markedly decreases both O_2^- generation and translocation of p47-*phox* and other cytosolic oxidase proteins *in vivo* (21,25). These observations support the conclusion that phosphorylation of p47-*phox* is a necessary event in the activation of the NADPH

oxidase *in vivo*. The role of p67-*phox* in the oxidase assembly process is not clear. It has been reported that p67-*phox* is phosphorylated during neutrophil activation (26-28), but this finding is controversial (29). Smith *et al.* (30) reported that p67-*phox* contained the NADPH binding site of the NADPH oxidase; however, this finding is not consistent with several studies reporting that flavocytochrome b contains the NADPH-binding site (31-33) as well as studies showing that purified flavocytochrome b itself can generate O_2^- when it is relipidated in a specific phospholipid mixture (34). Rac has been shown by several groups to bind to p67-*phox* (35-37). In addition, p67-*phox* Src homology 3 (SH3) domains have been shown to play an important role in NADPH oxidase assembly (38). Thus, p67-*phox* may function mainly as a structural adapter protein to facilitate binding of various proteins to the assembling oxidase complex.

Although the NADPH oxidase of human neutrophils is by far the best-characterized, this system is also important for host defense against infectious disease in essentially all vertebrates. In ruminants, leukocyte defense mechanisms are critically important in host defense responses to mucosal and respiratory infections (39). Yet, even though bovine and human neutrophils seem to play similar roles in host defense, a comparison of bovine with human cells reveals distinct differences that may reflect variations in regulatory mechanisms. For example, one of the most unusual features of bovine neutrophils is their lack of response to N-formyl chemotactic peptides, reportedly due to the absence of detectable N-formyl peptide receptors (40,41). Thus, the inflammatory responses of neutrophils from various species are not necessarily identical to those of human cells, and it is important to study neutrophils of different species, such as cattle, in order to provide a broader understanding

of neutrophil host defense processes in these animals. In addition, a comparison of the structure and function of neutrophil proteins from other species can lead to a better understanding of the role of these proteins in human cells.

Previously, NADPH oxidase proteins corresponding to human p47-*phox* and p67-*phox* have been identified in bovine cytosol, and Western blotting and functional comparisons have documented the conserved nature of these proteins between species (42-44). More recently, we cloned and sequenced the bovine flavocytochrome b proteins gp91-*phox* and p22-*phox* and showed that these proteins were homologous with the human counterparts (45); however, distinct differences between the bovine and human proteins were also observed, giving us clues to important structural/functional features. Homologues for human p47-*phox* and p67-*phox* have only been cloned in the murine system (46,47), although a partial clone for rabbit p67-*phox* was just recently reported by Pagano *et al.* (48). In any case, it is clear that important information about conserved structural and functional features can be obtained by sequence comparison of protein homologues across species.

In this study, I have cloned and sequenced the bovine NADPH oxidase cytosolic proteins, p47-*phox* and p67-*phox*, and provide a comparative analysis of the bovine sequences with their human and mouse homologues. In addition, I have expressed these proteins and utilized them in NADPH oxidase assays to demonstrate functional similarity between bovine and human oxidase proteins. These studies help to provide a better understanding of the structure and function of the NADPH oxidase in bovine cells but also contribute to our understanding of this host defense system in other vertebrates as well.

Materials and Methods

Materials

All chemicals used were of the highest purity grade available. Dulbecco's phosphate-buffered saline (DPBS) was purchased from GIBCO-BRL (Grand Island, NY). Alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphate substrate kits were from Bio-Rad (Hercules, CA). PCR Sequencing Kits were from US Biochemical (Cleveland, OH). SpinX columns were from Corning Costar Corp. (Cambridge, MA). Wizard Miniprep and Maxipreps were from Promega (Madison, WI). Magnagraph nylon membranes were from Micron Separations, Inc. (Westboro, MA). The bovine cDNA library used in these studies was a kind gift from Dr. Marcus Kehrli (USDA-ARS, Ames, IA). The library contained bovine bone marrow cDNA undirectionally ligated into the mammalian expression vector, pcDNA3.1, and transformed into *E. coli* TOP F' host cells. Oligonucleotide primers were synthesized by GIBCO-BRL and Oligos Etc., Inc. (Wilsonville, OR).

Polymerase chain reaction (PCR) and sequencing

PCR reactions were performed as described previously using a Stratagene RoboCycler 40 and Taq Plus enzyme (45). For cycle sequencing, PCR was performed on a Perkin Elmer 2400 thermocycler using Big Dye Terminators, and the samples were sequenced on an ABI 310 Genetic Analyzer (Perkin-Elmer ABI, Foster City, CA). The nucleotide sequence translation and analyses were performed with DNASTAR software.

Sequencing of bovine p47-phox and p67-phox

To sequence bovine p47-*phox*, primers were derived from consensus regions of human and murine p47-*phox* sequences. These primers were used in PCR to generate fragments of bovine p47-*phox* from a bovine bone marrow cDNA library. These gene fragments were sequenced via PCR sequencing using a PCR sequencing kit. Using the bovine sequences obtained from these gene fragments, we prepared additional primers for PCR and sequencing until a larger gene fragment was obtained. With forward primer 5'-CTTCATCCGTCACATCGCCC-3' and reverse primer 5'-GACCACCAGCCGTCCAGG-3', a 784 bp DNA fragment was amplified from a bovine bone marrow library cDNA. This PCR product was electrophoresed on an agarose gel, excised, and purified with a Spin-X column. The purified DNA was labeled with ^{32}P using a Decaprime II Kit (Ambion, Inc., Austin, TX), and the labeled probe was used to screen the bovine bone marrow library. For library screening, ~$5x10^5$ colonies were spread on Magnagraph 150mm nylon membranes placed on 150mm LB/amp agar plates, and the colonies were allowed to grow at 37° overnight. Duplicate lifts made from each plate were placed on LB/amp agar plates. The bacterial colonies were allowed to grow on the membranes ~4 hours at 37° and then lysed on Whatmann paper saturated with lysis buffer (2X SSC, pH 7.0, 5% SDS). The DNA was cross-linked to the membrane in a 700W microwave for 6 min/lift. Additional lifts were simultaneously microwaved by adding 2 min/additional lift. Using standard procedures (49), membranes were screened with the radiolabelled probe overnight, washed under moderate stringency, and placed under X-ray film. Areas containing hybridized colonies were pulled from the master plate, and replated for secondary screening. The procedure was repeated for

tertiary screening to insure single isolated clones were obtained. The isolated clones were analyzed by PCR with 5' and 3' vector primers to select clones with the largest inserts. Five clones were chosen, grown in LB/amp broth, and purified via Wizard Miniprep kits. Vector primers were used to sequence the 5' and 3' ends of each insert via cycle sequencing, as described below. Four of the clones contained the full-length p47-*phox* sequence, and two of these clones were sequenced completely in forward and reverse directions with an ABI 310 Genetic Analyzer to confirm the sequence.

To sequence bovine p67-*phox*, primers were derived from consensus regions of the human p67-*phox* sequence (the murine sequence was not reported at the time we began these experiments). These primers were used in PCR reactions to generate fragments of bovine p67-*phox* from the bovine bone marrow cDNA library. These gene fragments were sequenced via PCR sequencing using a PCR sequencing kit. Using the bovine sequences obtained from these gene fragments, we prepared additional primers for PCR and sequencing until a larger gene fragment was obtained. With two of the primer pairs (forward primer 5'-AGCCAGTGGTGATCCCTGTG-3' with reverse primer 5'-TTGAGTGTGTAGGCTT-GGGG-3' and forward primer 5'-CTTTGACTCAGCTTCGAGGG-3' with reverse primer 5'-GGTTCAAGGTAGTTGCAGGG-3), two gene fragments of 500 bp and 596 bp were obtained, respectively. Both fragments were purified, labeled with ^{32}P as described above, and simultaneously used to probe the bone marrow library in the same manner as described for p47-*phox*. From this screening, we selected 8 clones. These clones were amplified, purified, and sequenced as described above. Sequencing with vector primers revealed that 3 of the clones contained the complete p67-*phox* sequence, and two of these clones were

sequenced in forward and reverse directions completely with an ABI 310 Genetic Analyzer to confirm the sequence.

Expression of Bovine p47-phox and p67-phox

Phagemid clones containing the p47-*phox* and p67-*phox* sequences were amplified, purified by Wizard Maxiprep, and used to transform K562 erythroleukemia cells (ATCC # CCL-243) following the method of Ulrich and Ley (50). Briefly, K562 cells were grown to log phase in complete media (RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and L-glutamine). The cells were harvested, washed with RPMI-1640, and resuspended in RPMI 1640. Five hundred μl of cells (~$8.0x10^6$ cells) were mixed in a 0.2 mm BTX cuvette (BTX, San Diego, CA) with 100 μl of the DNA solution containing the phagemid, carrier DNA (sonicated herring sperm DNA), and Hebs buffer (20 mM Hepes pH 7.05, 137 mM NaCl, 0.7 mM NaH_2PO4). The cells were electroporated with a BTX Electrocell Manipulator set at 500V and 400 μF capacitance. The transfected cells were transferred to a tissue culture flask containing 10 ml of complete media and incubated in 7.5% CO_2 and 96% humidity at 37°C. After 48 hours, 1.8 μg/ml geneticin (G418) was added to select transfectants.

Partial purification of recombinant bovine NADPH oxidase proteins

Transfected and control cells were harvested by centrifugation at 250 x g for 10 minutes. The cells were washed three times in DPBS and resuspended at $1x10^8$ cells/ml in sonication buffer (10 mM Hepes pH 7.5, 10 mM NaCl, and 100 mM KCl) supplemented with protease inhibitors (3.3 μM chymostatin, 1.5 μM pepstatin, 0.15 μM aprotinin, 20 μM

leupeptin, and 1 μM EGTA). Cells were sonicated with four 5 second bursts on a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and centrifuged at 1000 x g to remove nuclei and cell debris. The supernatant was then centrifuged at 170,000 x g for 30 min with a Beckman Optima ultracentrifuge to remove all particulate material. The supernatant (i.e., cytosol) was stored at –80°C until use.

Due to low expression levels, the recombinant bovine NADPH oxidase proteins were partially purified using a modification of the methods of Leto and coworkers (51). For p67-*phox*, cytosol from 2 x 10^8 K562 cells transfected with bovine p67-*phox* was diluted to 50 ml with cold, sterile distilled water and filtered through a 0.2 μm pore filter. The diluted cytosol was applied to a BioRad Q2 anion exchange column that was equilibrated with 10 mM Bis-Tris propane buffer, pH 7.0, containing 0.1 mM DTT. After washing with equilibration buffer, the column was eluted with a linear gradient of 0-0.5 M NaCl in equilibration buffer. Fractions were screened for the presence of p67-*phox* by dot-blotting and Western blotting with anti-p67-*phox* antibody, as described below. Selected fractions containing p67-*phox* were concentrated to approximately 0.3 times their original volume using centrifugal concentrators.

For p47-*phox*, cytosol from 2 x 10^8 K562 cells transfected with bovine p47-*phox* was diluted and filtered, as described above. In this case, the diluted cytosol was applied to a 10 ml MacroPrep CM column (Bio-Rad) equilibrated with 5 mM potassium phosphate buffer (pH 7.0) and eluted with a linear gradient of 0-0.3 M NaCl in equilibration buffer. Selected fractions were concentrated to approximately 1/10 their original volume and screened for the presence of p47-*phox* by Western blotting. Fractions containing the highest levels of

recombinant p47-*phox* and p67-*phox* were used in functional assays as described below.

Cell-free NADPH oxidase reconstitution assay

Amphiphile-activated NADPH oxidase activity was measured spectrophotometrically in a heterologous cell-free NADPH oxidase assay system consisting of neutrophil membranes, threshold levels of cytosol, and recombinant p47-*phox* and p67-*phox* (52,53). Briefly, 200 µl reactions containing cell-free assay buffer (10 mM potassium phosphate, pH 7.2, 130 mM NaCl, 2 mM EGTA, 4 mM $MgCl_2$, 10 µM FAD, 2 mM NaN_3, 50 µM cytochrome c, and 10 µM GTPγS), 4 x 10^6 cell equivalents of human neutrophil membranes, 2 x 10^5 cell equivalents/ml of human neutrophil cytosol, and recombinant human or bovine p47-*phox* and p67-*phox* were incubated with of 40 µM arachidonic acid for 5 minutes, followed by the addition of 200 µM NADPH to initiate the reaction. The change in absorbance at 550 nm was measured continuously over 15 minutes at 25°C in a Molecular Devices THERMOmax microtiter plate reader, and actual rates of superoxide production were calculated using an extinction coefficient of 1.85 mM^{-1} cm^{-1} for cytochrome c. Duplicate reactions containing 100 µg/ml superoxide dismutase (SOD) were conducted in parallel and subtracted from the results to obtain only SOD-inhibitable activity. Recombinant human p47-*phox* and p47-*phox*, prepared as described previously (54), were used at 5 and 6 µg/well, respectively. Bovine recombinant proteins were added from the partially purified peak fractions, as described above (30 µl and 15 µl of the peak fractions from purification of recombinant bovine p47-*phox* and p67-*phox* were used in each reaction, respectively). The use of threshold levels of human neutrophil cytosol to provide essential

factors for NADPH oxidase activity besides p47-*phox* and p67-*phox* (e.g., Rac) has been established previously as a the standard approach in this type of analysis (52,53).

Electrophoresis and Western blot analysis

Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7-18% polyacrylamide gradient gels and transferred to nitrocellulose membrane, as described previously (55). For reference, prestained molecular weight standards (BRL, Bethesda, MD) were included on all gels. Western blots were probed with rabbit polyclonal anti-p67-*phox* or anti-p47-*phox* (54), followed by Bio-Rad alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody. The blots were developed using a Bio-Rad alkaline phosphatase development kit. Image analysis for molecular weight determination was performed with as IS-1000 Alpha Imager digital imaging system and Alpha Image software (Alpha Innotech, San Leandro, CA).

Results

Sequencing of bovine p47-phox and p67-phox

To sequence the bovine p47-*phox* gene, oligonucleotide primers were synthesized to consensus regions of the human and murine p47-*phox* nucleotide sequences as described under Materials and Methods. These primers were used to amplify a 784 bp DNA fragment of bovine p47-*phox*. The fragment was gel purified and labeled with ^{32}P as described under Materials and Methods, and used to screen a bovine bone marrow cDNA library by a standard hybridization assay. Four of the clones contained the full-length p47-*phox* gene

sequence, and two of these clones were sequenced completely (both clones gave the same sequence). For sequence verification, the two clones were sequenced twice from both strands.

The cDNA insert sequenced to obtain the p47-*phox* gene was 1324 nucleotides long, and contained an ORF of 1176 bases encoding 392 amino acids, which is two residues longer than the human and murine homologues. The calculated molecular weight of bovine p47-*phox* is 45,349 Da, which is slightly larger than that calculated for the human (44,686 Da) or murine (44,675 Da) proteins. Bovine p47-*phox* has a calculated pI of 9.2, which is similar to that calculated (both were 9.1) or experimentally determined (56,57) for human and murine p47-*phox*. The complete nucleotide and amino acid sequences for bovine p47-*phox* are shown in Figure 1. At the nucleotide level, bovine p47-*phox* has 85.2 and 80.1% identity with human and murine p47-*phox*, respectively. A comparative alignment between the bovine, human, and murine p47-*phox* amino acid sequences is shown in Figure 2. Comparison of the bovine p47-*phox* amino acid sequence with the other known p47-*phox* sequences demonstrated a high degree of similarity with the human (84.7%) and murine (82.2%) homologues.

To sequence the bovine p67-*phox* gene, oligonucleotide primers were synthesized to consensus regions of the human p67-*phox* nucleotide sequence as described under Materials and Methods. These primers were used to amplify 500 and 596 bp DNA fragments of bovine p67-*phox*. These fragments were gel purified, labeled with ^{32}P, and used to screen a bovine bone marrow cDNA library with a standard hybridization assay as described under Materials and Methods. Two clones containing the full-length p67-*phox* gene were selected and

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tgcagccgagcc**atg**gggggaccacttcatccgccacattgccctcctgggcttcgagaagcgcttc    66
            M  G  D  H  F  I  R  H  I  A  L  L  G  F  E  K  R  F

gtccccagccagcactacgtctacatgttcctggtgaaatggcaggacctgtctgagaaagtggtc    132
 V  P  S  Q  H  Y  V  Y  M  F  L  V  K  W  Q  D  L  S  E  K  V  V

tatcggcgcttcaccgagatctacgaattccacaaaatcttaaaggagatgtttcctattgaggcg    198
 Y  R  R  F  T  E  I  Y  E  F  H  K  I  L  K  E  M  F  P  I  E  A

ggggacatcaacccagagaataggatcatcccgcacctgccagccccgcggtggtatgatgggcag    264
 G  D  I  N  P  E  N  R  I  I  P  H  L  P  A  P  R  W  Y  D  G  Q

cgggtggccgagagccgccagggcactcttaccgagtactgcagcacgctcatgagcctgcctgtc    330
 R  V  A  E  S  R  Q  G  T  L  T  E  Y  C  S  T  L  M  S  L  P  V

aagatctcccgctgcccgcacctcctcaacttcttcaaggtgcgccccgacgacctcaagctcccc    396
 K  I  S  R  C  P  H  L  L  N  F  F  K  V  R  P  D  D  L  K  L  P

acagacagccaggtgaaaaagccagagacatacctgatgcccaaagatggcaagaacaacgctgcg    462
 T  D  S  Q  V  K  K  P  E  T  Y  L  M  P  K  D  G  K  N  N  A  A

gacatcacgggccccatcatcctgcagacgtaccgcgccatcgctgactacgaaaagggctcgagt    528
 D  I  T  G  P  I  I  L  Q  T  Y  R  A  I  A  D  Y  E  K  G  S  S

tcccaaatggctctggccacgggcgacgtggtggacgtcgtggagaagaacgagagcggctggtgg    594
 S  Q  M  A  L  A  T  G  D  V  V  D  V  V  E  K  N  E  S  G  W  W

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gatgaagccgaggacccagaacccaactatgcaggtgagccctacatcaccatcaaagcctacact    726
 D  E  A  E  D  P  E  P  N  Y  A  G  E  P  Y  I  T  I  K  A  Y  T

gctgtactggaggatgagatatccttggaggagggtgaagccattgaggtcattcataagctcctg    792
 A  V  L  E  D  E  I  S  L  E  E  G  E  A  I  E  V  I  H  K  L  L

gatggctggtgggtcatcaggaaagaagacgtcacaggttactttccatccatgtacctgcagaag    858
 D  G  W  W  V  I  R  K  E  D  V  T  G  Y  F  P  S  M  Y  L  Q  K

gcagggcaggacgtagcccaggccaaaagccagatcaagagccgggggggcgccaccccgcaggtcg    924
 A  G  Q  D  V  A  Q  A  K  S  Q  I  K  S  R  G  A  P  P  R  R  S

tccatccgcaatgctcacagcatccaccagcggtcacggaagcgcctcagccaggacacctatcgg    990
 S  I  R  N  A  H  S  I  H  Q  R  S  R  K  R  L  S  Q  D  T  Y  R

cgcaacagcgtccgttttatgcagcagcgccgccaccagcggccggagccacagcgctccaggagc   1066
 R  N  S  V  R  F  M  Q  Q  R  R  H  Q  R  P  E  P  Q  R  S  R  S

gccctgagggagcagcagcaacccaagaccgagcgccccaagccgcagccggccgtgccccccagg   1132
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cccagcgcagacctcatcctgcaccgctgcagcgagagcaccaagcggaagctggcctctgccgtc   1198
 P  S  A  D  L  I  L  H  R  C  S  E  S  T  K  R  K  L  A  S  A  V

**tga**ggccgcctccatttgatgctctcctcacccccagcccctaaacctgtacatacgtgtatagag   1264
**STOP**
agcctgaggttagcacccccctggccccgcccacccagctgttcgataccccttaataaacgttgctt   1330

ggagggaaaaaaaaaaaaaa                                                  1337
```

Figure 1: Nucleotide and Predicted Amino Acid Sequence of Bovine p47-*phox*. The predicted amino acid sequence is given in single letter code. This sequence data was deposited in GenBank under accession No. AF079302.

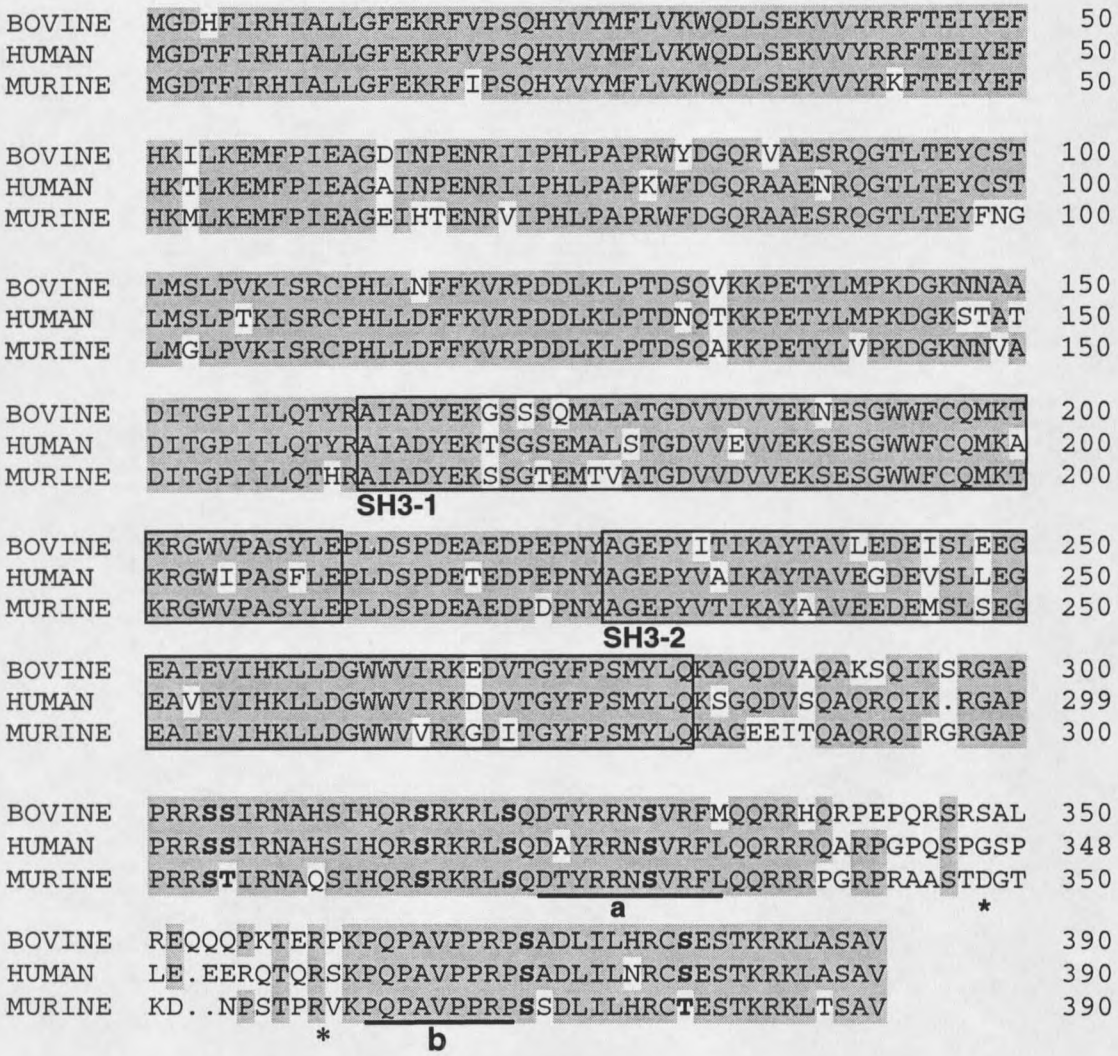

Figure 2: Comparison of bovine p47-*phox* amino acid sequence with the human and murine sequences. The predicted amino acid sequences for bovine, human, and murine p47-*phox* were aligned using MEGALIGN software (DNAStar, Madison, WI). Consensus amino acids among all sequences are shaded in gray. Both SH3 domains are shown in boxes, and the locations of consensus sites of phosphorylation between all homologues are indicated in bold. Putative consensus sites of phosphorylation postulated for human p47-*phox,* but not present in one or both of the other homologues, are indicated by a star (*). The putative p67-*phox*/flavocytochrome b binding region (a) (54,76) and proline-rich, p67-phox SH3 domain binding site (b) (72,81) are underlined. See text for further details.

sequenced completely (both clones gave the same sequence). For sequence verification, the two clones were sequenced twice from both strands.

The cDNA insert sequenced to obtain the p67-*phox* gene was 1863 nucleotides long, and contained an ORF of 1581 bases that encoded a protein 527 amino acids long. This sequence is slightly longer than that of human (526 residues) and murine (525 residues) p67-*phox*. The calculated molecular weight of bovine p67-*phox* is 59,667 Da, which is very close to that calculated for the human (59,765 Da) and murine (59,490) homologues. Bovine p67-*phox* has a calculated pI of 5.7, which is slightly lower than that calculated for human (6.0) and murine (6.4) p67-*phox*. The complete nucleotide and amino acid sequences for bovine p67-*phox* are shown in Figure 3. At the nucleotide level, bovine p67-*phox* has 88.3 and 81.3% identity with human and murine p67-*phox*, respectively. A comparative alignment between the bovine, human, and murine p67-*phox* amino acid sequences is shown in Figure 4. Comparison of the bovine p67-*phox* amino acid sequence with the other known p67-*phox* sequences demonstrated considerable similarity with the human (87.9%) and murine (82.9%) homologues.

Expression of bovine p47-phox and p67-phox

To verify function of bovine p47-*phox* and p67-*phox* encoded by the cDNAs identified above, we used the selected phagemid clones isolated from our cDNA library to transform K562 erythroleukemia cells, as described under Materials and Methods. Western blotting of cytosol from the transfected cells with polyclonal antibodies prepared against recombinant human proteins showed that we could express both recombinant bovine p47-*phox* and p67-

```
tgaaggcgggtttggggtggcccggctggggagacacgggcttgccctctcccccggtct   60

ccaggcagccctcccggtttgcacctgacc**atg**tccctggccgaggccatcagcctctgg  120
                              M  S  L  A  E  A  I  S  L  W

aatgaaggagtgctggcagctgacaagaaggactggaagggagccctggacgccttcact  180
 N  E  G  V  L  A  A  D  K  K  D  W  K  G  A  L  D  A  F  T

ggggtacaggacccccactccaggatctgcttcaatgtgggctgcatatacacgattctg  240
 G  V  Q  D  P  H  S  R  I  C  F  N  V  G  C  I  Y  T  I  L

gggaacctgccggaggcggagaaggccttcaccaaaagcattaaccgagacaagcacttg  300
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gcagtgtcctacttccaacgagggatgctctattaccagatggagaaatatgattccgcc  360
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 I  K  D  L  K  E  A  L  T  Q  L  R  G  N  Q  L  I  D  Y  K

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gctcagctggtcaagaaggattacctaggcaaggccacggtggtggcctctgtggtagac  720
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acctccctggagtctgatatcccagctcccccgagttccagtgctcctggaagaccccag 1080
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ttgtcaccaggtcagaaaggaaaagaagagcccaagcaggaaatcaagctcagtgtcccc 1140
 L  S  P  G  Q  K  G  K  E  E  P  K  Q  E  I  K  L  S  V  P

aagtcctacacactcaaggtgcattacaagtacacggtggtcatggagactcagttcagg 1200
 K  S  Y  T  L  K  V  H  Y  K  Y  T  V  V  M  E  T  Q  F  R
```

```
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 L  P  Y  S  Q  V  R  D  M  V  A  K  K  L  D  L  L  P  E  H

actaagctgagctaccgtcgtcaggacagcaatgagctggtgccccctttcagaattcagc 1320
 T  K  L  S  Y  R  R  Q  D  S  N  E  L  V  P  L  S  E  F  S

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 V  G  D  Q  G  F  P  D  E  P  E  E  S  K  K  S  D  A  N  N

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 Q  T  T  E  P  E  L  K  E  G  S  K  V  V  A  L  F  S  Y  E

gctacccagccagaagacctggagttcctggaaggggacgtcatcctggtgatatcaacg 1560
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tgagagacacctctggaattattaattcatccattaaagtattaatccatcatcaagtaa 1800


tattcatgaaataaatattaattcatttaactcttccattaaaacttaacctattagaca 1860

atg                                                            1863
```

Figure 3: Nucleotide and Predicted Amino Acid Sequence of Bovine p67-*phox*. The predicted amino acid sequence is given in single letter code. This sequence data was deposited in GenBank under accession No. AF079303

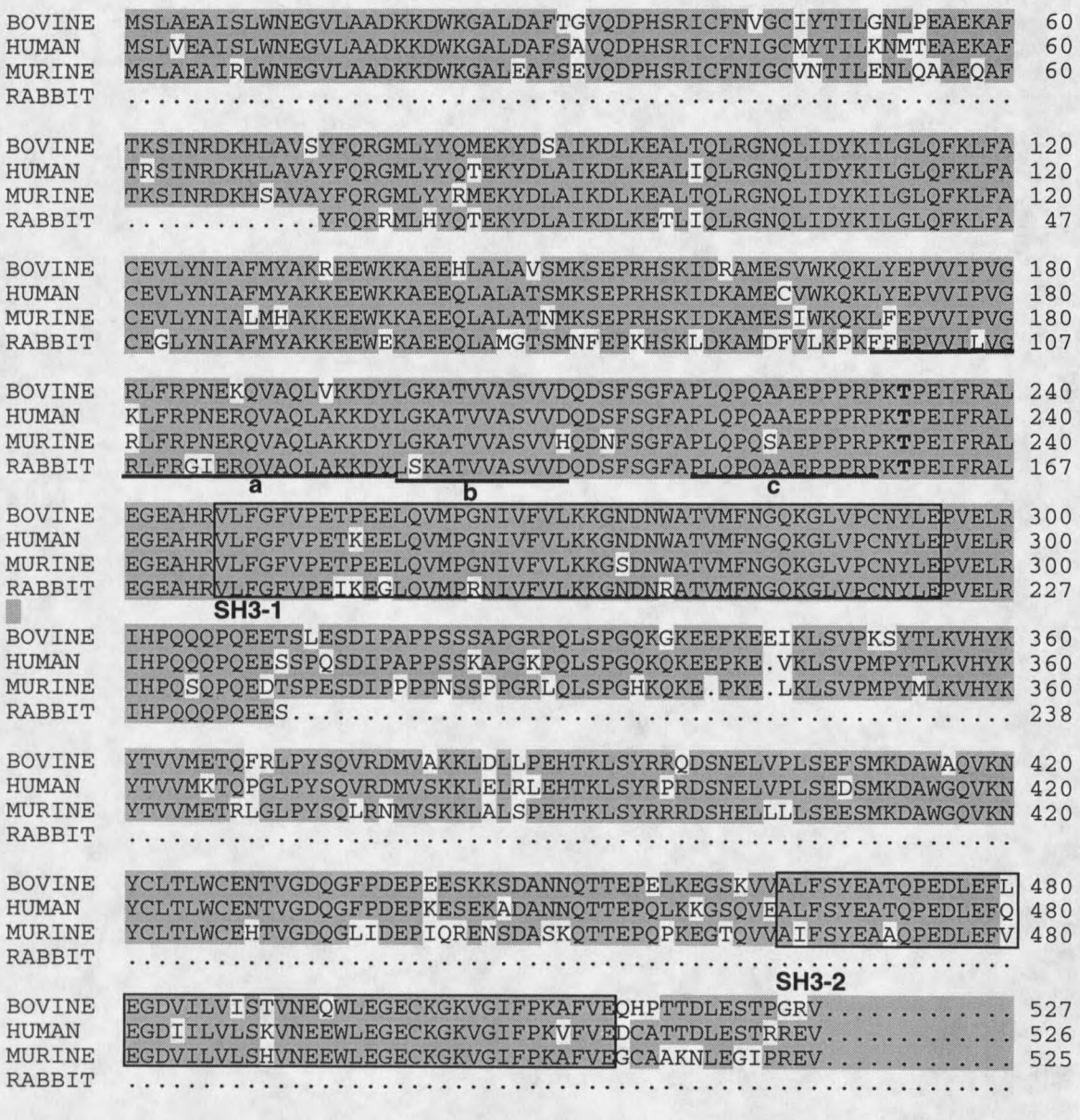

Figure 4: Comparison of Bovine p67-*phox* Amino Acid Sequence with Human and Murine Sequences. The predicted amino acid sequences for bovine, human, murine, and partial rabbit p67-*phox* were aligned using MEGALIGN software. Consensus amino acids among all sequences are shaded in gray, and the location of a putative PAK phosphorylation sites is indicated in bold. Both SH3 domains are shown in boxes. The putative Rac binding region (a) (28), activation domain (b) (78), and proline-rich, SH3 domain binding site (c) (72,81) are underlined. See text for further details.

phox and that the recombinant proteins were similar in size to the native proteins present in bovine and human neutrophil cytosol (Figure 5). These results are consistent with previous analyses of bovine cytosol (42-44). Image analysis of Western blots was used to determine actual molecular weights and showed that recombinant bovine p47-*phox* ran as an ~48 kDa protein, which is almost identical to native bovine and human p47-*phox* (both ~ 49 kDa). The slight differences observed in mobility are most likely to be due to differences in the amount of protein loaded in each lane. These results are consistent with previous comparisons of bovine and human cytosolic p47-*phox* (43,44). Recombinant bovine p67-*phox* ran as an ~64 kDa protein, while human p67-*phox* ran as an ~67 kDa protein, supporting previous reports that the bovine homologue was ~63-65 kDa (58).

Functional analysis of recombinant bovine p47-phox and p67-phox

Recombinant bovine p67-*phox* and p67-*phox* expressed in K562 cells was concentrated and used in cell-free NADPH oxidase assays to establish functional activity of the recombinant proteins. As shown in figure 6, recombinant bovine p47-*phox* and p67-*phox* proteins were functionally active in reconstituting NADPH oxidase activity in assays that contained human neutrophil membranes and threshold levels of cytosol (see Materials and Methods). These results demonstrate that the bovine homologues can functionally substitute for human p47-*phox* and p67-*phox*, further confirming the highly conserved nature of the proteins making up the NADPH oxidase (44,45).

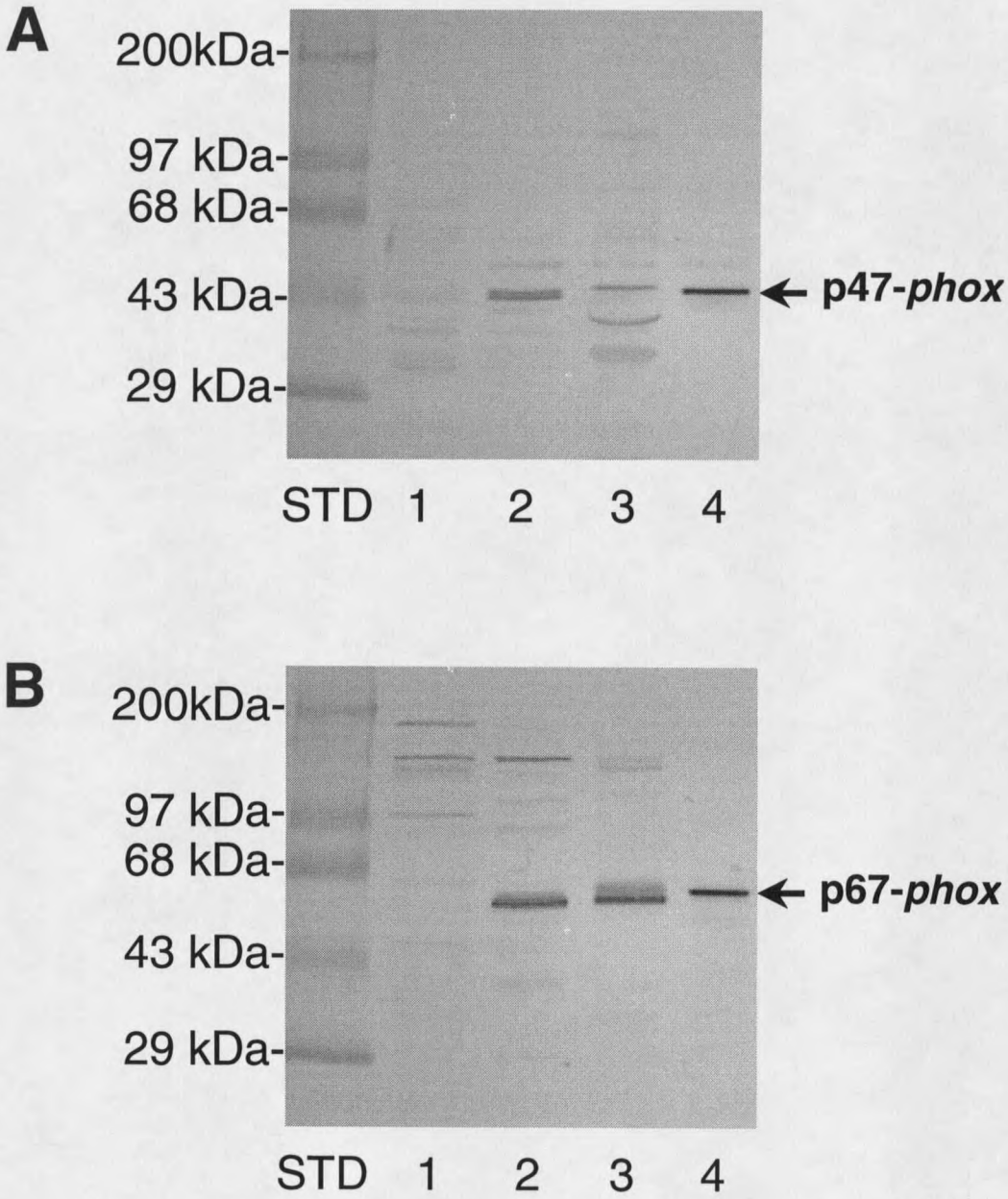

Figure 5: Western Blot Analysis of Recombinant Bovine p47-*phox* and p67-*phox*. Wild-type K562 cell cytosol (lane 1, both panels), recombinant bovine p47-*phox* (lane 2, panel A) or p67-*phox* (lane 2, panel B), bovine neutrophil cytosol (lane 3, both panels), and human neutrophil cytosol (lane 4, both panels) were analyzed by SDS-PAGE and Western blotting with rabbit polyclonal anti-human p47-*phox* (panel A) and rabbit polyclonal anti-human p67-*phox* (panel B), as described under Materials and Methods. Prestained molecular weight standards are shown in the first lane of both blots (STD). Representative of 3 separate experiments.

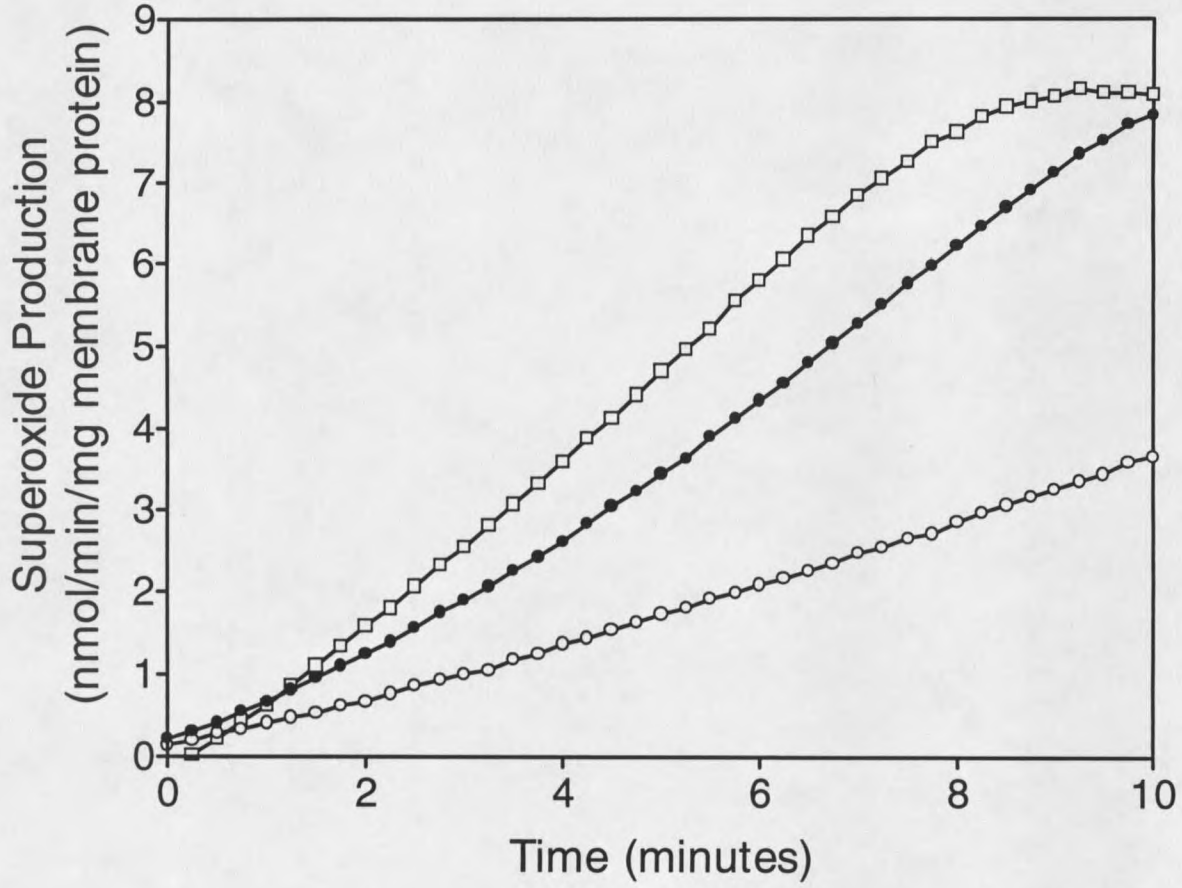

Figure 6: Functional Analysis of Recombinant Bovine p47-*phox* and p67-*phox*. SOD-inhibitable superoxide production was measured spectrophotometrically in a heterologous cell-free NADPH oxidase assay system as described under Materials and Methods. The results are expressed as nmol O_2^-/min/mg membrane protein for assays consisting of human neutrophil membranes and threshold levels of cytosol only (○), human neutrophil membranes, threshold levels of cytosol, and recombinant human p47-*phox* and p67-*phox* (□), and human neutrophil membranes, threshold levels of cytosol, and recombinant bovine p47-*phox* and p67-*phox* (●). Representative of 3 separate experiments.

Discussion

The phagocyte NADPH oxidase is a multi-protein enzyme complex that plays an essential role in host defense system [reviewed in (1,2)]; however, the transfer of electrons from NADPH to molecular oxygen *in vivo* occurs only after the translocation of the cytosolic components (p40-*phox*, p47-*phox*, p67-*phox*, and Rac2) to the membrane. Understanding the interaction of flavocytochrome b with these cytosolic NADPH oxidase proteins will be essential to our understanding of how this system assembles and is regulated. Since the functional role of the NADPH oxidase appears to be conserved across species, we propose that important information about conserved structural features the NADPH oxidase proteins can be obtained through comparison of homologues from various species. Determining the structure and functional characteristics of these homologues is also informative in defining key functional regions that may help define the role of these proteins in the human system.

In the present study, we report the cloning and sequencing of two bovine NADPH oxidase cytosolic proteins, p47-*phox* and p67-*phox*. In addition, we have expressed these proteins and shown that they are functional and can substitute for their human counterparts in a cell-free NADPH oxidase assay system. Although there is a high degree of homology in both of these protein sequences across all of the species analyzed to date, there are also distinct differences between homologues that may provide clues relative to various structural and functional features of these proteins. Interestingly, the overall level of homology among all known p47-*phox* and p67-*phox* sequences was somewhat lower than that observed between bovine flavocytochrome b and flavocytochrome b from other species (45). This

might be expected, given the central role flavocytochrome b plays in the actual electron transfer process (12).

The bovine p47-*phox* sequence contains a predicted ORF of 1176 nucleotides and encodes a protein of 392 amino acids, which is 2 amino acids longer than the human and murine homologues. The predicted molecular weight for this open reading frame is 45.3 kDa, which is slightly lower than observed by SDS-PAGE and Western blot analysis (~48.2 kDa). This relative difference between calculated and observed molecular weights is similar to that found with the human homologue (44.7 versus 49.2 kDa, respectively). Comparison of the human and bovine p47-*phox* amino acid sequences revealed that there was a high degree of divergence among all species in the C-terminal region of p47-*phox*. Consequently, while the amino acid sequence is relatively conserved throughout other regions of the protein, the sequence identity of residues 340-361 (human residues 339-359 and murine residues 340-359) falls to <36% between species.

Previous studies on human p47-*phox* have demonstrated that p47-*phox* is phosphorylated on a number of residues during NADPH oxidase activation (20-23). The phosphorylation of p47-*phox* has been proposed to neutralize a highly cationic region of p47-*phox* encompassing residues 314-347, initiating a conformational change within the protein and allowing the protein to interact with the membrane and/or target proteins (59-61). In human neutrophils stimulated with PMA or *f*MLF, phosphorylation was shown to occur in a number of discrete steps on at least seven serine residues and was closely correlated with activation of the oxidase (20). Recently, El Benna *et al.* (22) reported that p47-*phox* isolated from PMA-stimulated human neutrophils was phosphorylated on serines 303, 304, 320, 328,

345, and 348 and at least one of the serines at 359, 370, and 379. In comparison, bovine p47-*phox* contains serines at all of these sites, except for the residues corresponding to human p47-*phox* Ser348 and 359. These two serines are also not conserved in the murine p47-*phox* sequence, suggesting that phosphorylation of these residues in human p47-*phox* may not play an important role in NADPH oxidase activation. Consistent with recent studies by Faust *et al.* (23) showing that phosphorylation of Ser379 played a key role in p47-*phox* translocation to the membrane and NADPH oxidase activity, this serine and flanking consensus phosphorylation site were conserved among all three p47-*phox* homologues. Using two-dimensional phosphopeptide mapping, El Benna *et al.* (62) postulated that p47-*phox* was phosphorylated by protein kinase C, protein kinase A, and mitogen-activated protein kinase (MAPK). Based on sequence homology, these authors proposed the sequence around Ser345 and Ser348 represented a consensus sequence for phosphorylation by MAPK (62). However, this consensus sequence was not conserved in murine p47-*phox* (46). Furthermore, analysis of bovine p47-*phox* shows that this consensus site is also not conserved in the bovine sequence, supporting the conclusion of Leusen *et al.* (4) who proposed that p47-*phox* is not a MAPK substrate.

SH3 domains were originally identified as highly conserved, non-catalytic regions of the Src family of protein tyrosine kinases [reviewed in reference (63)] but have recently been shown to regulate protein-protein binding interactions in a number of systems (64,65). In addition, SH3 domains can also mediate localization of proteins to the sub-membranous cytoskeleton during the transduction of extracellular signals into the cell (65-67). The intermolecular association of NADPH oxidase components also appears to be modulated in

part by SH3 domain interactions, which facilitate the assembly process (38,68). SH3 domains recognize proline-rich binding motifs, and it has been proposed that SH3-mediated interactions aid in the stabilization of a cytosolic complex of NADPH oxidase proteins in resting neutrophils (38,69,70). In addition, SH3-mediated interactions appear to play a role in targeting protein-protein interactions during the oxidase assembly process (38,70,71). Human p47-*phox* and p67-*phox* both have two SH3 domains and an additional cytosolic oxidase protein, p40-*phox*, has one SH3 domain.

Like the human and murine homologues, bovine p47-*phox* also contains two SH3 domains as well as a proline rich motif near the carboxyl-terminus of the protein. The first SH3 domain, consisting of residues 163-211, shares 81.6 and 85.7% identity with the human and murine sequences, respectively. Residues 227-281 represent the second SH3 domain, and show 85.5% identity with this domain in both human and murine proteins. The C-terminal proline-rich region of p47-*phox* corresponding to residues 360-371 of human p47-*phox* (residues 361-372 in bovine p47-*phox*) was identical among all sequences, and this region of p47-*phox* is known to play a key role in binding a p67-*phox* SH3 domain (72,73). Thus, it would be expected that this region, which represents a consensus SH3 domain-binding site (74), is highly conserved across species. Recently, we found that both p67-*phox* and flavocytochrome b utilized a common binding site in p47-*phox* (residues 323-332), presumably at distinct stages of the activation process (54). These residues are located within a highly cationic p47-*phox* domain that also contains multiple potential phosphorylation sites (75). During activation, phosphorylation and subsequent conformation changes in p47-*phox* appear to result in exposure of this domain for binding to other oxidase

proteins (54,71). As shown in figure 2, this region of p47-*phox* is almost identical among all species. Thus, the high level of conservation in this region of p47-*phox* homologues from different species is supportive of previous biochemical data in showing a critical function for this domain in the human neutrophil NADPH oxidase assembly process (54,75,76).

The bovine p67-*phox* sequence contains a predicted ORF of 1581 nucleotides and encodes a protein of 527 amino acids, which is 1 and 2 amino acids longer than the human and murine homologues, respectively. The predicted molecular weight for this open reading frame is 59.7 kDa, which is slightly lower than that observed by SDS-PAGE and Western blot analysis (~64 kDa). Based on primary sequence comparison, bovine p67-*phox* has a calculated molecular weight that is almost identical to human and murine p67-*phox*. Thus, the amino acid differences between these two homologues must impart some alternative structural characteristics that result in the incongruent migration patterns on SDS-PAGE (i.e., bovine p67-*phox* runs as an ~64 kDa protein, while human p67-*phox* runs as an ~67 kDa protein). Interestingly, close inspection of the blot of p67-*phox* from bovine cytosol suggests that possibly two isoforms of p67-*phox* are present in bovine cytosol, one at ~64 kDa and one at ~67 kDa, the size observed for native human p67-*phox*. Further studies are currently in progress to investigate the possibility of p67-*phox* isoforms.

Comparison of the bovine p67-*phox* amino acid sequence with the human, murine, and partial rabbit sequences demonstrated a high level of identity, although the human and bovine sequences showed the highest degree of homology (87.9%). As with all other p67-*phox* homologues, bovine p67-*phox* has two SH3 domains, and the location and size of these domains appears to be conserved across species. The first p67-*phox* SH3 domain is located

at residues 247-295 and is highly conserved, differing by only one amino acid between the human and murine proteins (98% homology) and by only 5 residues when compared to rabbit p67-*phox* (90% homology). The second p67-*phox* SH3 domain is located at bovine p67-*phox* residues 465-513 and is also highly conserved among species (87.8% homology when compared to human or murine p67-*phox*). In addition to the SH3 domains, p67-*phox* contains a proline-rich SH3 domain-binding motif (residues 219-231) that has been proposed to mediate binding to the second SH3 domain of p47-*phox* during NADPH oxidase assembly (38,77). Comparison of p67-*phox* homologues shows that this region is identical across all species, supporting its putative role in NADPH oxidase function. Recently, Han *et al.* (78) used truncation mutations to identify an "activation domain" in human p67-*phox*. This domain spans p67-*phox* residues 199-210, and site-directed mutation of single amino acids in this region showed that p67-phox with a V204A mutation was completely inactive (78). Comparison among all p67-*phox* homologues shows that this region is identical between human and bovine proteins and is only different by one residue in murine and rabbit p67-*phox*. In all cases, however, Val204 was conserved, supporting its putative role in NADPH oxidase activation.

The interaction between p67-*phox* and Rac is essential for NADPH oxidase activation (35,79), and appears to be mediated primarily by binding of Rac to the amino-terminal region of p67-*phox* (residues 1-199) (35), although Faris *et al.* (80) recently found that the C-terminus of p67-*phox* may also play a role in stabilizing Rac binding. Residues 1-199 of bovine p67-*phox* are highly homologous with those of the human (89.4% identical) and murine (86.9% identical) homologues. Recently, Ahmed *et al.* (28) further narrowed

down the location of the Rac binding site to residues 171-199 of p67-*phox*, a region that is almost identical between bovine and human p67-*phox*, with only 3 conservative substitutions between the two proteins. In addition, Ahmed *et al.* (28) reported that p67-*phox* contained a cryptic p21-activated kinase (PAK) phosphorylation site located at Thr233. This residue is conserved among all p67-*phox* homologues, as is consistent with its proposed functional role in the NADPH oxidase. In addition, this site is located adjacent and downstream of the highly conserved proline-rich region (residues 219-231), which may bind to the C-terminal SH3 domain, resulting an intramolecular interaction that conceals the PAK phosphorylation site until activation-induced unfolding of p67-*phox* (28). Note, however, that PAK-mediated phosphorylation of p67-*phox* has only been demonstrated *in vitro*, and further studies *in vivo* will be necessary to verify if this event plays an actual functional role for in the NADPH oxidase.

In summary, we have cloned and sequenced two essential bovine NADPH oxidase cytosolic proteins, p47-*phox* and p67-*phox*. Furthermore, comparison of these bovine sequences with other p47-*phox* and p67-*phox* sequences previously reported has allowed us to further revise our understanding of the key structural features of these proteins. This information will be useful in studying the NADPH oxidase system in cattle as well as contribute to our understanding of the structure/function of human p47-*phox* and p67-*phox* and their roles in the assembly and activation of the O_2^--generating NADPH oxidase in phagocytic leukocytes.

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CONCLUSION

In cattle, neutrophils play an essential role in the cellular host defense, and defective neutrophil function has been associated with many disease states. A key component in host defense is the NADPH oxidase system associated with the neutrophil membrane. Activation of the NADPH oxidase generates reactive oxygen species which function to destroy microbial invaders, as well as induce inflammatory processes. A better understanding of the role of the NADPH oxidase in the bovine neutrophil is important both for animal welfare and in the prevention of economic loss associated with bovine disease.

The sequencing of the bovine flavocytochrome b proteins, gp91-*phox* and p22-*phox* revealed a high degree of homology with their counterparts in other species; however there were also important differences that may relate to differences in cell function among the various species. In this project, I have sequenced the bovine NADPH oxidase cytosolic proteins, p47-*phox* and p67-*phox*. My results show that these proteins are also highly homologous to the human, mouse, and rabbit homologues. Note, however, the overall levels of homology between the bovine cytosolic NADPH oxidase proteins and those in other species were slightly lower than those observed for the flavocytochrome b proteins (1). The expression of these proteins in a mammalian cell enabled additional confirmation of this conservation in structure, as recombinant bovine p47-*phox* and p67-*phox* cytosolic proteins were able to assemble with flavocytochrome b in human neutrophil membranes to produce O_2^- in a cell-free assay. The identification of the conserved regions within p47-*phox* and p67-*phox* confirmed reports of key structural features in the human and murine proteins.

However, the differences between the bovine p47-*phox* and p67-*phox* proteins and these proteins in other species may be equally important in understanding the role of these cytosolic proteins in NADPH oxidase activation.

The activation of the NADPH oxidase for microbial destruction as well as the deactivation of this system to avoid host injury are not well understood. Production of the individual NADPH oxidase components and their use in *in vitro* assays will further contribute to our understanding of the association of these proteins and their relationship with other components in resting and activated cells. Antibodies produced from these proteins can be used to follow these proteins in experimental settings. The ability to manipulate oxidase activity while controlling inflammation would have a profound effect on the treatment of disease.

The data presented in this thesis represent an important advance in our understanding of the defense mechanisms of bovine neutrophils and provide insight to the neutrophil's role in bovine inflammatory disease. Clearly, better understanding of these host defense mechanisms and their relationship to bovine health will contribute to the development of treatments for the prevention of disease in cattle.