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Flagellin and lipopolysaccharide stimulate the MEK-ERK signaling pathway in chicken heterophils through differential activation of the small GTPases, Ras and Rap1

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Abstract

The TLR agonists, flagellin (FLG) and lipopolysaccharide (LPS) stimulate functional activation and cytokine gene expression via the extracellular signal regulated kinase 1/2 (ERK1/2) MAP kinase cascade. However, the upstream mechanisms of these signaling events remain unknown. In mammals, the small GTP-binding protein Ras mediates ERK1/2 activation through activation of downstream effectors Raf-1-MEK1/2-ERK1/2 in response to a variety of stimuli. It is not clear whether this classic Ras cascade plays a role in TLR signaling in avian cells. In the present study, we investigated the role of Ras in FLG- and LPS-mediated signaling in ERK activation in chicken heterophils. Treatment of heterophils with LPS caused a rapid (within 5 min) activation of Ras-GTP. The role of Ras activation in LPS-induced stimulation of ERK1/2 was corroborated when the specific Ras inhibitor, FTI-277, inhibited ERK1/2 activation. The classic Ras-mediated pathway of ERK1/2 activation by LPS was confirmed when the specific Raf-1 inhibitor, GW 5074, and the MEK1/2 inhibitor, U0126, both reduced ERK activation by 51–60%. Of more interest was that treatment of the heterophils with FLG did not activate Ras-GTP. Likewise, neither FTI-277 nor GW 5074 had any effect on FLG-mediated activation of ERK1/2. Another small GTPase, Rap1, has been shown to play a role in mammalian neutrophil function. Using a Rap1-GTP pull-down assay, we found that FLG stimulation, but not LPS, of avian heterophils induced a rapid and transient Rap1 activation. Rap1 has been shown to activate the ERK1/2 via a different Raf family member B-Raf whose downstream effector is MEK1/2. We show here that FLG stimulation of heterophils induces the phosphorylation of Rap1. The FLG induction of the Rap1 → B-Raf → MEK1/2 → ERK1/2 cascade was confirmed by the reduction of ERK1/2 activation by the specific Rap1 inhibitor (GGTI-298) and U0126. The results demonstrate that for the first time that the small GTPase Ras family is involved in TLR signaling of avian heterophils with the TLR agonists LPS (Ras) and FLG (Rap1) inducing differential signaling cascades to activate the downstream ERK MAP kinase.
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Keywords: Ras; Rap1; Flagellin; LPS; Toll-like receptors; Chickens; Heterophils; Innate immunity; Signal transduction

1. Introduction

Recognition of potential pathogenic microbes by the innate immune system is the function of a class of cellular receptors known as the pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs). The innate system uses these germ-line encoded receptors to recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns [PAMPs]) of infectious microbes (Janeway and Medzhitov, 2002; Nau et al., 2002). TLR bind PAMPs via their leucine-rich repeat domain (LRR) (Akira and Hemmi, 2004). LRRs are found in diverse proteins and are always implicated in ligand recognition and signal transduction (Akira, 2003). Upon ligand binding, the conserved intracellular Toll-like/interleukin-1 receptor (TIR) domain of the TLR transfers danger signals into the cytosol by recruiting and activating a cascade of adaptor and effector proteins that initiate microbicidal killing mechanisms, the production of pro- and/or anti-inflammatory cytokines, and up-regulation of co-stimulatory molecules required for antigen presentation to the acquired immune system (Underhill and Ozinsky, 2002; Kopp and Medzhitov, 2003). The TLR-receptor superfamily represents an evolutionarily conserved signaling

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system that is a decisive determinant of the innate immune and inflammatory responses.

In chickens, seven orthologues of human TLRs have been found (two genes corresponding to mammalian TLR1/6/10, TLR2 type 1, TLR type 2, TLR3, TLR4, TLR5, and TLR7) (Iqbal et al., 2005; Philbin et al., 2005; Kogut et al., 2005). Although no homologue of TLR9 has been found in the chicken, stimulation of either avian macrophages or peripheral blood monocytes with different CpG dinucleotides, signaling mediated by TLR9, induced a differential cytokine gene expression and nitric oxide production in avian macrophages (He et al., 2003; He and Kogut, 2003). Polymorphonuclear leukocytes (PMNs) are vital cellular components of innate immunity, and function by killing pathogenic microbes following phagocytosis. The primary PMN in poultry is the heterophil, the avian equivalent to the mammalian neutrophil. Like the neutrophil, avian heterophils are involved in the phagocytosis and killing of invading microbes. We have found that heterophils constitutively express all seven known chicken TLRs and when stimulated with specific TLR agonists functionally activate heterophil oxidative burst and degranulation (Kogut et al., 2005) and induce up-regulation of pro-inflammatory cytokines and inflammatory chemokines (Kogut et al., 2006).

The signaling pathways of TLR have been comprehensively studied in mammals, but not in lower vertebrates although the signaling proteins that are activated are also conserved across species (Dalpke and Heeg, 2002; O'Neill, 2002; Akira, 2003). We have begun to address this issue in the avian system. In mammals, stimulation of TLRs results in the downstream activation of the cytoplasmic portion of the TLR, the so-called Toll/IL-1 receptor (TIR) domain which then recruits MyD88/IRAK/TRAF6 and activates the mitogen activated protein kinase (MAPK) superfamily cascade (O'Neill, 2002; Dalpke and Heeg, 2002; Akira, 2003) and the transcription factors, NF-κB and AP-1 that leads to the expression of genes that participate in the innate immune response including pro-inflammatory cytokines. The MAP kinase (MAPK) superfamily of serine/threonine kinases consists of at least three distinct families: p38, extracellular signal-regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK) that play a major role in cellular activation of a variety of cell types (Cobb and Goldsmith, 1995). In mammalian cells, the phosphorylation of the MAPK superfamily has been established as the hallmark of cellular activation following TLR engagement (O'Neill, 2002). We have found that heterophils, when stimulated with specific TLR agonists, activate the p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK signaling cascades leading to the up-regulation of pro-inflammatory cytokine gene expression (Dong et al., 2002).

The ERK pathway is activated in response to signals from cell surface receptors (Marshall, 1995) and is crucial for immune cell activation leading to transcriptional regulation of cytokine genes, translational regulation, and other effector functions (Karin, 1995). Signals to the ERK pathway are generated by a variety of upstream stimuli that converge on the Ras family of small G proteins (Campbell et al., 1998). Ras induces its function through activating downstream effectors of the Raf-MAK-ERK cascade (Marshall, 1996; Rausch and Marshall, 1999).

Our laboratory is interested in inflammatory mediators that initiate the activation of avian innate host defenses; specifically those mediated by heterophils. By defining the intracellular signals that are transduced in heterophils following pathogen recognition, we can potentially develop agents that regulate the physiological innate host defenses in poultry.

The precise mechanisms of FLG and LPS activation of ERK1/2 in chicken heterophils have not been fully elucidated. There are no reports about the relevance of Ras in either TLR5 or TLR4-mediated signal transduction in chicken heterophils. In this study, we investigated the role of small G proteins in the activation of ERK1/2 by FLG and LPS in avian heterophils.

2. Materials and methods

2.1. Experimental chickens

Leghorn chickens (Hy-Line W-36) were obtained on the day-of-hatch from a commercial hatchery (Hy-Line International, Bryan, TX) and placed in floor pens on pine shavings. Birds were provided water and a balanced, unmedicated ration *ad libitum*. The feed ration contained or exceeded the levels of critical nutrients recommended by the National Research Council (1994).

2.2. Reagents

Ultra-pure lipolysaccharide (from *Salmonella minnesota*; LPS) and flagellin (from *Salmonella typhimurium*; FLG) were purchased from InVivoGen (San Diego, CA) and prepared in sterile physiological water as per manufacturer's instructions. The phospho-B-Raf (Ser445) antibody was obtained from Cell Signaling Technology (Beverly, MA). The inhibitors GW 5074 (for inhibiting Raf-1) and U0126 (for inhibiting MEK1/2) were purchased from Tocris Bioscience (Ellisville, MO, USA). PD98059 (for ERK1/2) and FTI-277 (for inhibiting Ras) were purchased from Sigma–Aldrich Inc. (St. Louis, MO). GGTI-298 was obtained from CalBiochem (San Diego, CA). All of the inhibitors were dissolved in DMSO and stock solutions were stored at 4 °C until used. Working concentrations of the inhibitors were prepared in RPMI 1640 tissue culture medium from the stock solutions. The final concentration of DMSO in the experiments was less than 0.5%.

2.3. Isolation of peripheral blood heterophils

Avian heterophils were isolated from the peripheral blood of day-old chickens as described previously (Kogut et al., 2005). Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood was mixed with 1% methylcellulose (25 centiposes; Sigma Chemical Co., St. Louis, MO) at a 1.5:1 ratio and centrifuged at 25 × *g* for 30 min. The serum and buffy coat layers were retained and suspended in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co.). This suspension was layered over a discontinuous Ficoll–Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over