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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO ??, pp. 1–xxx, ???? ??, 2009
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Molecular Basis for E-cadherin Recognition by Killer Cell Lectin-like Receptor G1 (KLRG1)*[S]

Received for publication, June 29, 2009, and in revised form, July 30, 2009 Published, JBC Papers in Press, August 4, 2009, DOI 10.1074/jbc.M109.038802

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The killer cell lectin-like receptor G1, KLRG1, is a cell surface receptor expressed on subsets of natural killer (NK) cells and T cells. KLRG1 was recently found to recognize E-cadherin and thus inhibit immune responses by regulating the effector function and the developmental processes of NK and T cells. E-cadherin is expressed on epithelial cells and exhibits Ca^{2+}-dependent homophilic interactions that contribute to cell-cell junctions. However, the mechanism underlying the molecular recognition of KLRG1 by E-cadherin remains unclear. Here, we report structural, binding, and functional analyses of this interaction using multiple methods. Surface plasmon resonance demonstrated that KLRG1 binds the E-cadherin N-terminal domains 1 and 2 with low affinity (K_d ~7–12 μM), typical of cell-cell recognition receptors. NMR binding studies showed that only a limited N-terminal region of E-cadherin, comprising the homodimer interface, exhibited spectrum perturbation upon KLRG1 complex formation. It was confirmed by binding studies using a series of E-cadherin mutants. Furthermore, killing assays using KLRG1⁺NK cells and reporter cell assays demonstrated the functional significance of the N-terminal region of E-cadherin. These results suggest that KLRG1 recognizes the N-terminal homodimeric interface of domain 1 of E-cadherin and binds only the monomeric form of E-cadherin to inhibit the immune response. This raises the possibility that KLRG1 detects monomeric E-cadherin at exposed cell surfaces to control the activation threshold of NK and T cells.

AQ: A

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Natural killer (NK)[3] cells play a critical role in the innate immune system because of their ability to kill other cells. For example, NK cells can kill virus-infected cells and tumor cells without presensitization to a specific antigen, and they produce various cytokines, including interferon-γ and tumor necrosis factor-α (1). NK cells are controlled by both inhibitory and activating receptors that are expressed on their surfaces (2). The killer cell Ig-like receptor, Ly49, CD94/NKG2, and paired Ig-like type 2 receptor families include both inhibitory and activating members and thus are designated as paired receptor families. On the other hand, some inhibitory receptors, including KLRG1 (killer cell lectin-like receptor G1), and activating receptors, such as NKG2D, also exist. The integration of the signals from these receptors determines the final functional outcome of NK cells.

AQ: B

These inhibitory and activating receptors can also be divided into two structurally different groups, the Ig-like receptors and the C-type lectin-like receptors, based on the structural aspects of their extracellular regions. The Ig-like receptors include killer cell Ig-like receptors and the leukocyte Ig-like receptors, and the C-type lectin-like receptors include CD94/NKG2(KLRD/KLRC), Ly49(KLRA), NKG2D(KLRK), NKR-P1(KLRB), and KLRG1. Many of these immune receptors recognize major histocompatibility complex class I molecules or their relatives (2–4), but there are still many orphan receptors expressed on NK cells. KLRG1 was one such orphan receptor; however, E-cadherin was recently found to be a ligand of KLRG1 (5, 6). Although major histocompatibility complex-receptor interactions have been extensively examined, the molecular basis of non-major histocompatibility complex ligand-receptor recognition is poorly understood.

KLRG1 is a type II membrane protein, with one C-type lectin domain in the extracellular region, one transmembrane region, and one immunoreceptor tyrosine-based inhibitory motif. KLRG1 is expressed on a subset of mature NK cells in spleen, lungs, and peripheral blood during normal development. KLRG1 expression is induced on the surface of NK cells during viral responses (7, 8). NK cells expressing KLRG1 produce low levels of interferon-γ and cytokines and have a slow *in vivo* turnover rate and low proliferative responsiveness to interleukin-15 (9). Furthermore, KLRG1 is recognized as a marker of some T cell subsets, as follows. KLRG1 defines a subset of T cells, short lived effector CD8 T cells (SLECs), which are mature effector cells that express high levels of KLRG1 and cannot be differentiated into long lived memory CD8 T cells. In addition, memory precursor

* This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, and the Japan Bio-oriented Technology Research Advancement Institute (BRAIN).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.

[1] Supported by the Canadian Cancer Society/National Cancer Institute of Canada and the Canadian Research Chair program.

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[3] The abbreviations used are: NK, natural killer; SLEC, short lived effector CD8 T cell; MES, 4-morpholineethanesulfonic acid; SPR, surface plasmon resonance; AUC, analytical ultracentrifugation; HSQC, heteronuclear sequential quantum correlation; PE, phycoerythrin.

Enzyme-mediated protein refolding†‡

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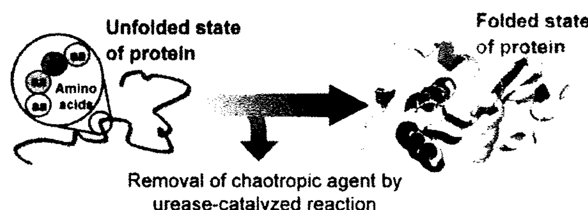
Received (in Cambridge, UK) 6th August 2009, Accepted 1st October 2009
First published as an Advance Article on the web 16th October 2009
DOI: 10.1039/b916225a

We employed a urease-catalyzed reaction to gradually remove a high concentration of a chaotropic agent (urea) from a denatured protein solution and demonstrated that efficient protein refolding can be achieved by the urease-catalyzed reaction, without large-volume dilution.

Progress in genetic engineering has enabled the production of a wide variety of recombinant proteins using host cells. The production of natural and genetically engineered proteins in a host cell, however, often leads to insoluble aggregation of inactive or denatured proteins; often in so-called inclusion bodies. There has been an ongoing search for an effective method for large-scale refolding of recombinant proteins from inclusion bodies (containing over-expressed and misfolded proteins), which will benefit the bioengineering industry.[1] Refolding of proteins from inclusion bodies, in principle, starts with solubilization of the inclusion bodies using an aqueous solution containing a high concentration of a chaotropic agent (8 M urea or 6 M guanidine HCl).[2] The gradual decrease in the concentration of the chaotropic agent in a solubilized protein solution then allows intra- and intermolecular interactions to occur and re-fold an unfolded protein. However, intermolecular interaction among proteins might cause misfolding and aggregation of proteins and produce an inactive protein. To date, many methods based on the above principle have been developed to achieve protein refolding with high renaturation efficiency and high renaturation yield. Among them, dilution is still the most widely used in the laboratory and in industry due to its simplicity and broad applicability. Large-volume dilution, including step-wise dilution, not only decreases the concentration of a chaotropic agent but also increases the distance between protein molecules thereby reducing intermolecular interactions. However, large-volume dilution has several drawbacks, especially in a large scale operation.[1] For example, it produces a large volume of protein solution with a low protein concentration, which requires protein condensation for storage and subsequent application. Furthermore, there are difficulties in uniform mixing in a large volume, resulting in further misfolding of the protein.

To overcome the above-mentioned drawbacks and achieve protein refolding, we employed a urease-catalyzed reaction to mediate protein refolding (Scheme 1). Urease catalyzes hydrolysis of urea to produce NH_3 and CO_2. The urease-catalyzed reaction can gradually remove urea from an unfolded protein solution and initiate protein renaturation. This method has two major advantages. First, urea was gradually and homogeneously removed from the protein solution; thus preventing the heterogeneous distribution of urea in a refolding solution. Secondly, a relatively high protein concentration can be maintained during the refolding process because no excessive dilution is required. To the best of our knowledge, this is the first report of protein refolding mediated by a urease-catalyzed reaction.

It has been previously reported that urease can hydrolyze high concentrations of urea (up to 4 M).[3] Prior to attempting urease-mediated protein renaturation, we explored the hydrolysis of urea at high concentrations. We confirmed that urease from jack beans can hydrolyze urea at concentrations up to 2 M. Although the urease-catalyzed reaction seems useful for the removal of urea from a refolding solution, the renatured-protein solution will contain urease as a contaminant if free urease is used. Therefore, urease was immobilized onto an NHS (*N*-hydroxysuccinimide)-activated Sepharose resin, which enables simple separation of the urease from a renatured protein. The hydrolysis of 2 M urea was investigated using the immobilized urease (Fig. 1). The hydrolysis rate of urea depended on the concentration of the immobilized urease. Urease at 1.0 mg ml^{-1} gradually reduced the urea concentration and completely removed urea in 24 h. The pH value of the solution drastically decreased within a couple of hours and reached pH ~9.5 at 24 h. Based on these investigations, urea decomposition was carried out using 1.0 mg ml^{-1} immobilized urease in the refolding experiments. Three different proteins were tested as model proteins to demonstrate



Scheme 1 Schematic illustration of the protein refolding mediated by the urease-catalyzed reaction.

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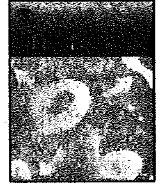
† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b916225a

Molecular and Cellular Endocrinology 300 (2009) 32–36

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce



Characterization of mice deficient in Melanocortin 2 receptor on a B6/Balbc mix background

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ARTICLE INFO

Article history:
Received 27 August 2008
Received in revised form 15 October 2008
Accepted 17 October 2008

Keywords:
Proopiomelanocortin (POMC)
Glucocorticoid (GC)
Melanocortin 2 receptor (MC2R)
Hypothalamic–pituitary–adrenal (HPA) axis
Familial glucocorticoid deficiency (FGD)
Renin–angiotensin–aldosterone system (RAAS)

ABSTRACT

We have previously reported that Melanocortin 2 receptor (MC2R$^{-/-}$) deficient mice on B6 N5 generations exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF) and lack of detectable levels of corticosterone, and reduced serum concentrations of aldosterone and epinephrine. All MC2R$^{-/-}$ mice on B6/N8 background die within 2 days after birth, while about half of the MC2R$^{-/-}$ mice on B6/Balbc mix background survived to adulthood. Both male and female MC2R$^{-/-}$ mice were fertile, suggesting that normal development and function of reproductive organs. MC2R$^{-/-}$ mice delivered from MC2R$^{-/-}$ dams failed to survive due to lung failure, suggesting that fetal or maternal corticosterone is essential for lung maturation. MC2R$^{-/-}$ mice failed to activate the hypothalamic–pituitary–adrenal axis in response to both immune and non-immune stimuli. MC2R$^{-/-}$ mice maintained glomerular structure and achieved electrolyte homeostasis by the activation of the renin–angiotensin–aldosterone system under low aldosterone and undetectable levels of corticosterone.
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1. Introduction

The body responds to stress by activation of the hypothalamic–pituitary–adrenal (HPA) axis and release of glucocorticoids (GCs) under the control of ACTH. ACTH secreted from the anterior pituitary is in turn regulated by hypothalamic corticotropin-releasing hormone (CRH) and Arginine vasopressin (AVP). This HPA axis is regulated by negative feedback exerted by serum cortisol levels on both the hypothalamus and the pituitary gland. ACTH is also the main regulator of adrenal cortical growth. Tissue specific post-translational cleavage of the prohormone, proopiomelanocortin (POMC) gives rise to bioactive peptides including melanotropic peptides, ACTH and several endorphins. We have previously generated mice with an inactivation mutation of the Melanocortin 2 receptor (MC2R) gene, and reported that MC2R on fifth generation of backcrossing to C57/BL6 (B6/N5) leads to neonatal lethality in about three-quarters of MC2R$^{-/-}$ pups, possibly due to hypoglycemia (Chida et al., 2007). Those surviving to adulthood exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF), lack of detectable levels of GC, and reduced serum concentrations of aldosterone and epinephrine (Chida et al., 2007).

GCs are secreted into the systemic circulation from the adrenal cortex and initiate a broad range of actions throughout the organism that regulate the function of multiple organ systems including the central nervous, endocrine, and immune systems. The physiological effects of GCs are mediated by intracellular glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) that function as ligand-dependent transcription factors (Mangelsdorf et al., 1995). Even though MR has a high affinity for GCs, the majority of the physiological effects of GCs are thought to be mediated via the GR, which is expressed more ubiquitously and is a stronger transcriptional activator. Physiologically, MR is thought to act primarily as a high affinity receptor for mineralocorticoids to control the sodium/potassium balance in the kidney and large intestine.

MC2R$^{-/-}$ mice are valuable for familial GC deficiency studies, and a unique animal model for investigation of the physiological functions of GC. The physiological role of GR and MR in vivo have been extensively studied in conditional KO mice (Tronche et al., 1999; Berger et al., 2006), due to the neonatal death of

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doi:10.1016/j.mce.2008.10.027

RhoH Plays Critical Roles in FcεRI-Dependent Signal Transduction in Mast Cells[1]

Hiroyo Oda,* Manabu Fujimoto,† Michael S. Patrick,*‡ Dai Chida,* Yoshinori Sato,* Yoshinao Azuma,‡ Hiroki Aoki,§ Takaya Abe,¶ Harumi Suzuki,[2]* and Mutsunori Shirai‡

RhoH is an atypical small G protein with defective GTPase activity that is specifically expressed in hematopoietic lineage cells. RhoH has been implicated in regulation of several physiological processes including hematopoiesis, integrin activation, and T cell differentiation and activation. In the present study, we investigated the role of RhoH in mast cells by generating RhoH knockout mice. Despite observing normal development of mast cells in vivo, passive systemic anaphylaxis and histamine release were impaired in these mice. We also observed defective degranulation and cytokine production upon FcεRI ligation in RhoH-deficient bone marrow-derived mast cells. Furthermore, FcεRI-dependent activation of Syk and phosphorylation of its downstream targets, including LAT, SLP76, PLCγ1, and PLCγ2 were impaired, however phosphorylation of the γ-subunit of FcεRI remained intact. We also found RhoH-Syk association that was greatly enhanced by active Fyn. Our results indicate that RhoH regulates FcεRI signaling in mast cells by facilitating Syk activation, possibly as an adaptor molecule for Syk. ***The Journal of Immunology*, 2009, 182: 957–962.**

RhoH is a newly identified hematopoietic small G protein, originally cloned as one of the genes frequently disrupted in diffuse large B cell lymphoma (1, 2). Because RhoH is defective in GTPase activity and thus constitutively active, the function of this protein was thought to be regulated by its expression level. Overexpression of RhoH inhibited RhoA/Rac/cdc42-dependent NF-κB activation in HEK293 cells (3), and it also inhibited SCF-mediated Rac1 activation in bone marrow progenitor cells (4). Knockdown of RhoH increased spontaneous LFA-1-mediated adhesion in Jurkat cells (5), and in vitro colony formation in bone marrow progenitor cells (4). Recent studies, however, have demonstrated that RhoH plays critical roles in T cell development (6, 7) by functioning as an adaptor for ZAP-70 in TCR signaling (7) via its tyrosine-phosphorylated ITAM-like motif (8). In the absence of RhoH, development of T cells in the thymus is impaired in both β-selection and positive selection, resulting in a severe reduction of mature peripheral T cells (6, 7). RhoH-deficient T cells showed defective phosphorylation of LAT and ERK upon TCR stimulation, indicating that RhoH is critical in TCR-dependent proximal signal transduction events. The precise function of RhoH in TCR signaling, however, remains controversial because there is a discrepancy in the phosphorylation status of ZAP-70 between two reports (6, 7). Furthermore, the physiological function of RhoH in other hematopoietic lineage cells is largely unknown.

Mast cells are widely distributed in the body and function as the primary effectors in immediate-type hypersensitivity reactions (9, 10). Mast cells recognize Ags via IgE and specific, high-affinity Fc receptors, termed FcεRI (11–13). FcεRI cross-linking triggers activation of Src family kinases Lyn and Fyn, and phosphorylation of ITAM motifs on the γ subunit of FcεRI complexes (14, 15). Subsequently, ZAP-70-related Syk kinase binds to phosphorylated ITAM motifs of the γ subunit and is thus activated by Src kinases (16–18). Activated Syk, in turn, phosphorylates LAT, LAT-2, and SLP-76 to form the signalosome, which transduces signals downstream, initiating Ca^{2+} mobilization, degranulation, and the expression of specific genes (19–21). The FcεRI-initiated signal cascade in mast cells is analogous to the TCR-initiated signal cascade in T cells, sharing many common molecules and features (14, 15, 22). This prompted us to investigate the function of RhoH in FcεRI signaling in mast cells.

In this study, we report the critical role of RhoH in mast cell activation. We established RhoH-deficient mice to unveil the physiological roles of RhoH in mast cells. RhoH-deficient mice showed impaired passive systemic anaphylaxis (PSA)[3] and histamine release upon challenge with the specific Ag. Our in vitro data showing impaired Syk activation with defective degranulation and cytokine production in $RhoH^{-/-}$ mast cells supports the observed in vivo phenotypes. Furthermore, we demonstrated that RhoH associates with Syk, and this interaction was greatly enhanced in the presence of constitutively active Fyn. These results suggest that RhoH acts as a positive regulator for FcεRI-mediated signal transduction by facilitating Syk activation.

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Received for publication January 16, 2008. Accepted for publication November 6, 2008.

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[1] This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology.

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[3] Abbreviations used in this paper: PSA, passive systemic anaphylaxis; BMMC, bone marrow-derived mast cell; HSA, human serum albumin; HA, hemagglutinin.

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Immunology Letters 124 (2009) 27–34

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Rac GTPases are involved in development, survival and homeostasis of T cells

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ARTICLE INFO

Article history:
Received 27 February 2009
Received in revised form 23 March 2009
Accepted 29 March 2009
Available online 5 April 2009

Keywords:
Rac
T cell survival and homeostasis

ABSTRACT

Rac GTPases consist of Rac1, 2 and 3, and each of them have redundant and differential functions. Rac1 is the most ubiquitously and abundantly expressed of the three and has been shown to work as a "molecular switch" in various signal transduction pathways. Although Rac1 and Rac2 are both activated by TCR ligation, little is known about the function of Rac GTPases in the development and activation of T cells. In order to investigate the precise function of Rac GTPases in T cells in vivo, we established dominant negative Rac1 transgenic (dnRac1-Tg) mice controlled by the human CD2 promoter. Total numbers of thymocytes of dnRac1-Tg mice were significantly decreased because of impaired transition from the CD4CD8 double negative stage to the CD4CD8 double positive (DP) stage. Although positive selection of CD4 single positive (SP) was not altered, positive selection of CD8-SP was slightly increased. On the contrary, the number of mature CD4-SP and CD8-SP cells in the spleen, mesenteric lymph nodes and peripheral blood was severely decreased in dnRac1-Tg mice. Proliferation of splenic CD4-SP cells upon TCR stimulation in vitro was unaltered, however, homeostatic proliferation of dnRac1-Tg splenic CD4-SP cells in lymphopenic mice was severely reduced. Finally, we found increased spontaneous apoptosis of DP thymocytes and mature T cells in dnRac1-Tg mice, possibly because of reduced phosphorylation of Akt with or without TCR stimulation. Collectively, the current results indicate that Rac GTPases are important in survival of DP thymocytes and mature T cells in vivo by regulating Akt activation.
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1. Introduction

Rac belongs to the Rho family of small guanosinetriphosphatases (GTPases), and is a critical signaling regulator acting as a molecular switch in mammalian cells [1–4]. Rac consists of three independent genes; Rac1 is expressed ubiquitously, Rac2 is expressed only in hematopoietic cells, and Rac3 is expressed mainly in the brain [1–5]. Rac1 regulates various cellular functions such as cellular growth, cytoskeletal rearrangement, and apoptosis [6–8]. Rac2 is important for superoxide production, phagocytosis by neutrophils, regulation of leukocyte lineage differentiation, regulation of B cell adhesion and immunological-synapse formation [9–11]. Rac3 is relevant to later events in the development of a functional nervous system [12]. Because Rac1 deficient mice die in utero [13], a conditional knockout strategy was applied for the analysis of its function. Distinct and critical roles of Rac1 and Rac2 in growth and engraftment of hematopoietic stem cells [5,14,15] as well as in B cell development [16] were reported by using conditional knockout mice for Rac1 on a $Rac2^{-/-}$ background.

However, little is known about the function of Rac GTPases in T cell development and activation. Rac2-deficient mice showed normal T cell development in the thymus, defective Th1 differentiation caused by decreased IFN-γ production [17], perturbed chemotaxis [18], and defective T cell activation accompanied by decreased ERK activation [19]. Overexpression of constitutively active mutants of Rac1 (L61Rac1 and L61Y40CRac1) induced pre-T cell differentiation and proliferation on a $RAG^{-/-}$ background [20], and conversion of positive selection to negative selection in the thymus [21]. The results indicates that Rac1 regulates the strength of TCR-mediated signal transduction. Very recently, mice with conditional disruption of both Rac1 and Rac2 in the thymus were reported and they showed reduced development of T cell and impaired activation with increased cell death [22,23].

Although Rac1 and Rac2 are abundantly expressed in the thymus, we found that Rac3 too was weakly expressed in the thymus. Because all three Rac GTPases are expressed in the thymus, we decided to establish transgenic mice with thymocyte-specific expression of a dominant negative mutant form of Rac1

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0165-2478/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.imlet.2009.03.014

Gasp, a Grb2-associating protein, is critical for positive selection of thymocytes

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Communicated by Tadamitsu Kishimoto, Osaka University, Osaka, Japan, July 31, 2009 (received for review June 11, 2009)

T cells develop in the thymus through positive and negative selection, which are responsible for shaping the T cell receptor (TCR) repertoire. To elucidate the molecular mechanisms involved in selection remains an area of intense interest. Here, we identified and characterized a gene product Gasp (Grb2-associating protein, also called Themis) that is critically required for positive selection. Gasp is a cytosolic protein with no known functional motifs that is expressed only in T cells, especially immature CD4/CD8 double positive (DP) thymocytes. In the absence of Gasp, differentiation of both CD4 and CD8 single positive cells in the thymus was severely inhibited, whereas all other TCR-induced events such as β-selection, negative selection, peripheral activation, and homeostatic proliferation were unaffected. We found that Gasp constitutively associates with Grb2 via its N-terminal Src homology 3 domain, suggesting that Gasp acts as a thymocyte-specific adaptor for Grb2 or regulates Ras signaling in DP thymocytes. Collectively, we have described a gene called Gasp that is critical for positive selection.

differentiation | signal transduction | T cell receptor | thymus

Development of conventional T cell receptor (TCR)-$\alpha\beta$ T cells in the thymus requires multiple stages defined by the expression pattern of CD4 and CD8 coreceptor molecules. The most immature $CD4^-CD8^-$ [double negative (DN)] thymocytes differentiate to the $CD4^+CD8^+$ [double positive (DP)] stage through the first selection process called β-selection (pre-TCR selection). These DP thymocytes are subjected to both positive and negative selection to become either class II MHC-restricted helper $CD4^+CD8^-$ [CD4-single positive (CD4-SP)] or class I MHC-restricted cytotoxic $CD4^-CD8^+$ (CD8-SP) cells (1). After receiving positive selection signals, DP thymocytes go through an intermediate $CD4^+CD8^{lo}$ stage, irrespective of their lineage decision (2). The fate of individual DP thymocytes is determined by the strength of affinity and longevity of interaction between their TCR and peptide:MHC ligand (3). Although it is known that strong TCR/ligand interaction leads to negative selection and weak association results in positive selection (4), how this quantitative difference of TCR interaction can be converted to the qualitative difference is not known. Therefore, it is important to investigate the difference in molecular mechanisms of positive and negative selection.

One of the widely accepted models for explaining the difference between positive and negative selection is differential MAPK activation (5). Initially, differential requirements for ERK in positive selection and JNK/p38 in negative selection were focused on (6). The guanine nucleotide exchange factor (GEF) Sos has dual activity for Ras and Rac, therefore it can activate both the ERK and JNK/p38 pathways. Recently, RasGRP, which is another GEF for Ras, was shown to be critical for positive but not negative selection (7). Furthermore, mice heterozygous for Grb2, which constitutively associates with Sos, showed inefficient JNK/p38 activation, but normal ERK activation (8). From these results, positive selection signals were thought to induce the RasGRP/Ras/ERK pathway, and negative selection signals were thought to induce the Grb2-Sos/Rac/JNK p38 pathway. The model that activation through RasGRP results in weak sustained ERK activation to induce positive selection, whereas activation through Sos induces strong temporary ERK activation leading to negative selection is still widely accepted (9). Recently, Daniels et al. (10) elegantly showed that positive selection signals induced subcellular compartmentalization of RasGRP/Ras/ERK to the Golgi membrane, whereas negative selection signals induced localization of Grb2-Sos/Ras/ERK to the plasma membrane. Furthermore, positive selector-induced ERK activation lasted longer in Golgi than in the plasma membrane. Therefore, subcellular compartmentalization of Ras-GEF and Ras upon TCR stimulation is now widely accepted to be the branch point of positive and negative selection (11).

To find novel genes involved in the positive selection of thymocytes, we tried to isolate unknown genes whose expression is highly restricted to the thymus, the site where selection takes place. We used EST databases and performed in silico cloning, the strategy successfully used for isolating various novel tissue or cell type-specific genes. We selected several "thymus-specific genes" by our own computer algorithm based on their thymus-restricted expression. Among these thymus-specific genes, we focused on one gene E430004N04Rik (GeneID 210757), mainly because of its exclusive expression in immature DP thymocytes. Because we found that this protein constitutively associates with Grb2, we called the gene Gasp (Grb2-associating protein). Gasp contains no known protein motifs or homology domain and has no known function, although it has well conserved orthologs in multiple vertebrates from fish to human. To elucidate the function of Gasp, we established *Gasp*-deficient mice and found that the gene is critical for positive selection but not for other TCR-mediated signaling events.

Results

Expression of Gasp Protein. To identify novel genes specifically expressed in the thymus, we used information from the National Center for Biotechnology Information Unigene database of expressed sequence tags (ESTs). Each Unigene cluster contains information about the number of EST clones from a tissue source. We searched Unigene clusters based on the proportion of clones derived from thymus and total number of clones from the thymus. Finally, we selected five genes as thymus-specific genes. The gene Gasp (E430004N04Rik, Themis, Tsepa) was one of these thymus-specific genes. We first examined the expression of Gasp in various

Author contributions: T.S. and H.S. designed research; M.S.P., H.O., K.H., Y.S., K.E., S.-i.I., M.S., and T.N. performed research; T.K. and T.A. contributed new reagents/analytic tools; M.S.P., H.O., K.E., S.-i.I., T.N., and H.S. analyzed data; and M.S.P. and H.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0908593106/DCSupplemental.

IMMUNOLOGY

Published December 15, 2008

JEM

ARTICLE

The Journal of Experimental Medicine

Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC

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Plasmacytoid dendritic cells (pDCs) are an important source of type I interferon (IFN) during initial immune responses to viral infections. In mice, pDCs are uniquely characterized by high-level expression of Ly49Q, a C-type lectin-like receptor specific for class I major histocompatibility complex (MHC) molecules. Despite having a cytoplasmic immunoreceptor tyrosine-based inhibitory motif, Ly49Q was found to enhance pDC function in vitro, as pDC cytokine production in response to the Toll-like receptor (TLR) 9 agonist CpG-oligonucleotide (ODN) could be blocked using soluble monoclonal antibody (mAb) to Ly49Q or H-2K^b. Conversely, CpG-ODN–dependent IFN-α production by pDCs was greatly augmented upon receptor cross-linking using immobilized anti-Ly49Q mAb or recombinant H-2K^b ligand. Accordingly, Ly49Q-deficient pDCs displayed a severely reduced capacity to produce cytokines in response to TLR7 and TLR9 stimulation both in vitro and in vivo. Finally, TLR9-dependent antiviral responses were compromised in Ly49Q-null mice infected with mouse cytomegalovirus. Thus, class I MHC recognition by Ly49Q on pDCs is necessary for optimal activation of innate immune responses in vivo.

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Abbreviations used: B6, C57BL/6; BAC, bacterial artificial chromosome; BST2, bone marrow stromal cell antigen 2; DOTAP, 1,2-dioleoyloxy-3-trimethylammonium-propane; ES, embryonic stem; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; mDC, myeloid DC; MCMV, mouse CMV, MFI, mean fluorescence intensity; mPDCA-1, mouse PDC antigen 1; ODN, oligonucleotide; pDC, plasmacytoid DC; SHP, Src homology phosphatase; TLR, Toll-like receptor.

Plasmacytoid DCs (pDCs) are potent antiviral effector cells that were originally identified by their plasma cell–like morphology and localization within the T cell zone of lymphoid tissue (1). Also termed type I IFN-producing cells, pDCs secrete more type I IFN on a per-cell basis than any other cell type (2–4). pDCs are especially important in controlling viral infections, a property highlighted by their selective expression of Toll-like receptor (TLR) 7 and TLR9 (5), which recognize single-stranded RNA and double-stranded DNA, respectively. pDCs do not express TLR2, TLR3, TLR4, and TLR5, explaining why they do not respond to common bacterial products recognized by other APCs.

pDCs represent a rare cell type constituting ~1% of bone marrow or splenic leukocytes and <0.5% of lymph node and peripheral blood leukocytes. However, their frequency varies between mouse strains with 129Sv mice possessing a significantly higher proportion of pDCs than other mouse strains (6). Mouse pDCs do not express the lineage markers CD19, CD3, DX5, CD14, or TER119 (7, 8). In addition to their selective pattern of TLR expression, pDCs and myeloid DCs (mDCs) are dissimilar in various other aspects. Unlike mDCs, pDCs are characterized by a CD11b$^-$B220$^+$Ly6C$^+$ phenotype (7). Like mDCs, pDCs express CD11c but they do so at a lower level (8). Resting pDCs have been referred to as immature APCs because they express only low levels of CD86 and class II MHC, and they display little or no endocytic

L.-H. Tai and M.-L. Goulet contributed equally to this paper.

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The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 13 3187-3199
www.jem.org/cgi/doi/10.1084/jem.20080718

Genes and Immunity (2008) 9, 214–223
© 2008 Nature Publishing Group All rights reserved 1466-4879/08 $30.00
www.nature.com/gene

ORIGINAL ARTICLE

Association of LILRA2 (ILT1, LIR7) splice site polymorphism with systemic lupus erythematosus and microscopic polyangiitis

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Leukocyte immunoglobulin-like receptors (LILRs) are inhibitory, stimulatory or soluble receptors encoded within the leukocyte receptor complex. Some LILRs are extensively polymorphic, and exhibit evidence for balancing selection and association with disease susceptibility. LILRA2 (LIR7/ILT1) is an activating receptor highly expressed in inflammatory tissues, and is involved in granulocyte and macrophage activation. In this study, we examined the association of LILRA2 and adjacently located LILRA1 with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and microscopic polyangiitis (MPA). Polymorphism screening detected a LILRA2 SNP (rs2241524 G>A) that disrupts splice acceptor site of intron 6. Case–control association studies on 273 Japanese SLE, 296 RA, 50 MPA and 284 healthy individuals revealed increase of genotype A/A in SLE (12.1%, odds ratio (OR) 1.82, 95% confidence interval (CI) 1.02–3.24, P = 0.041) and in MPA (16.0%, OR 2.52, 95% CI 1.07–5.96, P = 0.049) compared with healthy individuals (7.0%). The risk allele caused an activation of a cryptic splice acceptor site that would lead to a novel LILRA2 isoform lacking three amino acids in the linker region (Δ419–421). Flow cytometry indicated that this isoform was expressed on the surface of monocytes. These findings suggested that LILRA2 Δ419–421 isoform encoded by the splice site SNP may play a role in SLE and MPA.
Genes and Immunity (2008) 9, 214–223; doi:10.1038/gene.2008.5; published online 14 February 2008

Keywords: *LILRA2; splice acceptor site; polymorphism; systemic lupus erythematosus; microscopic polyangiitis*

Introduction

The family of leukocyte immunoglobulin (Ig)-like receptor (LILR, also known as LIR, immunoglobulin-like transcript (ILT), CD85 or monocyte/macrophage inhibitory receptor) consists of 13 member genes including two pseudogenes (LILRP1 and LILRP2).[1–3] LILRs can be divided into three groups. The first group (LILRA1, -A2, -A4, -A5 and -A6) delivers positive signals by pairing with the Fc receptor common γ-chain (FcRγ), which contains an immunoreceptor tyrosine-based activation motif. The second (LILRB1, -B2, -B3, -B4 and -B5) contains 2–4 immunoreceptor tyrosine-based inhibitory motif-like sequences within the cytoplasmic region and inhibits cell activation by recruiting Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) or SH2 domain-containing inositol phosphatase (SHIP). The third group, LILRA3 and LILRA5s, is thought to be secreted as soluble receptors.

The *LILR* gene family is located in the leukocyte receptor complex on human chromosome 19q13.4, which also contains a number of closely related Ig-like receptor genes such as killer cell Ig-like receptors (*KIRs*) and *FCAR*.[4–6] This region has been suggested to be one of the candidate susceptibility regions for systemic lupus erythematosus (SLE) by linkage analyses.[7,8] In the syntenic region of mice, proximal end of chromosome 7, activating and inhibitory paired Ig-like receptor A and B (*Pira*, *Pirb*), orthologs of human *LILR* genes, are located.[9,10] Mice lacking *Pirb* showed impaired maturation of dendritic cells (DCs) and increased Th2 responses.[11] Furthermore, it was reported that both PIR-A and -B proteins bind to various mouse MHC class I molecules, and *Pirb*-deficient mice showed exacerbated graft-versus-host disease.[12] These observations suggest that LILRs may be critically involved in the regulation of immune system in various aspects.

LILRA2 is an activation receptor broadly expressed on hematopoietic cells such as monocytes, macrophages, myeloid DCs, granulocytes and subset of NK cells.[13,14] It

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Received 6 September 2007; revised 17 January 2008; accepted 17 January 2008; published online 14 February 2008

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ScienceDirect

Journal of Virological Methods 149 (2008) 171–174

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Short communication

Homogeneous sugar modification improves crystallization of measles virus hemagglutinin

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Received 15 October 2007; received in revised form 19 December 2007; accepted 10 January 2008
Available online 4 March 2008

Abstract

Measles virus (MV) enters cells by binding to the signaling lymphocyte activation molecule (also called CD150) on the cell surface, and thus shows the lymphotropism and immunosuppressive effects. The head domain (residues Asp^{149} to Arg^{617}) of the MV hemagglutinin (MV-H), the attachment protein, was produced using a transient expression system in HEK293T cells. The purified MV-H protein was heterogeneous because of a variety of complex-sugar modifications. The complex-sugar-type MV-H was crystallized successfully, and the crystals belonged to the space group P41212 with the unit cell dimension of $a = b = 134$ Å, $c = 100$ Å, but diffracted only to 3.0 Å resolution. MV-H was also expressed in HEK293SGnTI(−) cells lacking the *N*-acetylglucosaminyltransferase I activity, which render N-linked glycans of the proteins restricted and homogeneous, producing the oligomannose, $Man_5GlcNAc_2$. The native and selenomethionyl derivative proteins of the oligomannose-type MV-H were crystallized, and the native crystals well diffracted to 2.6 Å resolution. Thus, homogeneous sugar modification may be useful for improved crystallization of heavily sugar-modified viral envelope proteins.
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Keywords: Measles virus; Hemagglutinin; Crystallization; Restricted *N*-glycosylation; HEK293S-GnTI(−) cells; HEK293T cells

Measles virus (MV) is an enveloped virus with a non-segmented negative-strand RNA genome and a member of the *Morbillivirus* genus in the *Paramyxoviridae* family (Lamb and Parks, 2007). MV continues to be a major killer of children, still responsible for 4% of deaths in children younger than 5 years of age worldwide (Bryce et al., 2005). MV infection causes profound immunosuppression, which makes measles patients susceptible to secondary infections accounting for high morbidity and mortality (Griffin, 2007).

MV possesses two envelope glycoproteins, the hemagglutinin (MV-H) and fusion protein, which are responsible for receptor binding and membrane fusion, respectively. Many paramyxoviruses use sialic acid as a receptor, whereas MV does not recognize sialic acid, and instead binds to the signaling lymphocyte activation molecule (SLAM, also called CD150), a membrane glycoprotein expressed on immature thymocytes, activated lymphocytes, macrophages and dendritic cells, to enter cells (Tatsuo et al., 2000; Yanagi et al., 2006). This explains both the tropism and immunosuppressive properties of MV (Yanagi et al., 2006).

The Edmonston strain of MV was isolated in 1954 from a patient with measles by using a primary culture of human kidney cells (Enders and Peebles, 1954). Currently used live attenuated MV vaccines, which are safe and very effective, were obtained by passaging this original isolate many times in a variety of cell types, including primary human kidney and amnion cells and chicken embryo fibroblasts. The Edmonston vaccine strain has acquired the ability to bind to CD46 expressed ubiquitously during the passage, although clinical isolates of MV do not usually use CD46 as a receptor (Yanagi et al., 2006).

Recently, several expression methods were developed to produce glycoproteins suitable for crystallization, which aim to achieve homogeneous sugar modification with or without the treatment with endoglycosidase H or related enzymes (Aricescu

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0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.01.008

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Biochimica et Biophysica Acta 1784 (2008) 621–628

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Serotriflin, a CRISP family protein with binding affinity for small serum protein-2 in snake serum

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Received 5 September 2007; received in revised form 18 December 2007; accepted 24 December 2007
Available online 5 January 2008

Abstract

Habu (*Trimeresurus flavoviridis*) serum contains 3 small serum proteins (SSP-1, SSP-2, and SSP-3) with molecular masses of 6.5 to 10 kDa. Gel filtration analysis showed that all the SSPs exist in high molecular mass forms of approximately 60 kDa in the serum. Ultrafiltration of Habu serum showed that SSPs dissociated from the complex below a pH of 4. An SSP-binding protein was purified from Habu serum by gel filtration, ion exchange, and reverse-phase HPLC. N-terminal sequencing yielded a 39-amino acid sequence, similar to the N-terminal region of triflin, which is a snake venom-derived Ca^{2+} channel blocker that suppresses smooth muscle contraction. The amino acid sequence of this protein, termed serotriflin, was established by peptide analysis and cDNA cloning. Serotriflin is a glycosylated protein and consists of 221 amino acids. Among the 3 SSPs, only SSP-2 formed a noncovalent complex with serotriflin. It was bound to triflin and serotriflin with high affinity, as evidenced by surface plasmon resonance. SSP-2 is considered to be a protein that prevents self injury by accidental leaking of venom into the blood.
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Keywords: CRISP; PSP94; SSP; Snake serum; Triflin; *Trimeresurus flavoviridis*

1. Introduction

The human prostatic secretory protein of 94 amino acids (PSP94) is a 10.7-kDa, nonglycosylated, cysteine-rich protein [1], and the homologous proteins have also been identified in several mammals [2–4] and ostrich [5]. In addition to the 10 conserved cysteine residues that form 5 disulfide bonds, there are only 16 amino acids conserved across all the mammalian proteins, suggesting that they evolved at a relatively rapid rate with few constraints of the selection pressure [6].

Recently, we isolated a novel low molecular mass protein from the serum of Habu (*Trimeresurus flavoviridis*) and termed this protein small serum protein (SSP) [7]. Analysis of the N-terminal residues indicated that SSP belongs to the PSP94 protein family, despite the low sequence homology, since the cysteine residues of these proteins were topologically similar. We also found 2 similar proteins (SSP-2 and SSP-3) in the same serum, and the first SSP was then renamed SSP-1. SSPs are the first PSP94 family proteins to be found in reptiles.

SSP-1 showed an inhibitory effect against brevilysin H6 [8], a snake venom metalloproteinase with weak hemorrhagic activity. SSP-3 also exhibited a slight inhibition of brevilysin H6. On the other hand, SSP-2 demonstrated a strong binding affinity for triflin, which was isolated from *T. flavoviridis* venom as the protein responsible for blocking smooth muscle contraction and belongs to the cysteine-rich secretory protein (CRISP) family [9]. This suggests that SSP-2 exists in snake blood for defense against the toxic effects of venom in case of accidental self-envenomation. To our knowledge, SSP-2 is the

Abbreviations: CRISP, cysteine-rich secretory protein; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; P_{60}, 60%; ammonium sulfate fraction; PSP94, prostatic secretory protein of 94 amino acids; PSPBP, PSP94-binding protein; SCP, sperm coating glycoprotein; SSP, small serum protein

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1570-9639/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2007.12.010

RhoH Plays Critical Roles in FcεRI-Dependent Signal Transduction in Mast Cells[1]

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RhoH is an atypical small G protein with defective GTPase activity that is specifically expressed in hematopoietic lineage cells. RhoH has been implicated in regulation of several physiological processes including hematopoiesis, integrin activation, and T cell differentiation and activation. In the present study, we investigated the role of RhoH in mast cells by generating RhoH knockout mice. Despite observing normal development of mast cells in vivo, passive systemic anaphylaxis and histamine release were impaired in these mice. We also observed defective degranulation and cytokine production upon FcεRI ligation in RhoH-deficient bone marrow-derived mast cells. Furthermore, FcεRI-dependent activation of Syk and phosphorylation of its downstream targets, including LAT, SLP76, PLCγ1, and PLCγ2 were impaired, however phosphorylation of the γ-subunit of FcεRI remained intact. We also found RhoH-Syk association that was greatly enhanced by active Fyn. Our results indicate that RhoH regulates FcεRI signaling in mast cells by facilitating Syk activation, possibly as an adaptor molecule for Syk. *The Journal of Immunology*, **2009, 182: 957–962.**

RhoH is a newly identified hematopoietic small G protein, originally cloned as one of the genes frequently disrupted in diffuse large B cell lymphoma (1, 2). Because RhoH is defective in GTPase activity and thus constitutively active, the function of this protein was thought to be regulated by its expression level. Overexpression of RhoH inhibited RhoA/Rac/cdc42-dependent NF-κB activation in HEK293 cells (3), and it also inhibited SCF-mediated Rac1 activation in bone marrow progenitor cells (4). Knockdown of RhoH increased spontaneous LFA-1-mediated adhesion in Jurkat cells (5), and in vitro colony formation in bone marrow progenitor cells (4). Recent studies, however, have demonstrated that RhoH plays critical roles in T cell development (6, 7) by functioning as an adaptor for ZAP-70 in TCR signaling (7) via its tyrosine-phosphorylated ITAM-like motif (8). In the absence of RhoH, development of T cells in the thymus is impaired in both β-selection and positive selection, resulting in a severe reduction of mature peripheral T cells (6, 7). RhoH-deficient T cells showed defective phosphorylation of LAT and ERK upon TCR stimulation, indicating that RhoH is critical in TCR-dependent proximal signal transduction events. The precise function of RhoH in TCR signaling, however, remains controversial because there is a discrepancy in the phosphorylation status of ZAP-70 between two reports (6, 7). Furthermore, the physiological function of RhoH in other hematopoietic lineage cells is largely unknown.

Mast cells are widely distributed in the body and function as the primary effectors in immediate-type hypersensitivity reactions (9, 10). Mast cells recognize Ags via IgE and specific, high-affinity Fc receptors, termed FcεRI (11–13). FcεRI cross-linking triggers activation of Src family kinases Lyn and Fyn, and phosphorylation of ITAM motifs on the γ subunit of FcεRI complexes (14, 15). Subsequently, ZAP-70-related Syk kinase binds to phosphorylated ITAM motifs of the γ subunit and is thus activated by Src kinases (16–18). Activated Syk, in turn, phosphorylates LAT, LAT-2, and SLP-76 to form the signalosome, which transduces signals downstream, initiating Ca^{2+} mobilization, degranulation, and the expression of specific genes (19–21). The FcεRI-initiated signal cascade in mast cells is analogous to the TCR-initiated signal cascade in T cells, sharing many common molecules and features (14, 15, 22). This prompted us to investigate the function of RhoH in FcεRI signaling in mast cells.

In this study, we report the critical role of RhoH in mast cell activation. We established RhoH-deficient mice to unveil the physiological roles of RhoH in mast cells. RhoH-deficient mice showed impaired passive systemic anaphylaxis (PSA)[3] and histamine release upon challenge with the specific Ag. Our in vitro data showing impaired Syk activation with defective degranulation and cytokine production in RhoH$^{-/-}$ mast cells supports the observed in vivo phenotypes. Furthermore, we demonstrated that RhoH associates with Syk, and this interaction was greatly enhanced in the presence of constitutively active Fyn. These results suggest that RhoH acts as a positive regulator for FcεRI-mediated signal transduction by facilitating Syk activation.

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Received for publication January 16, 2008. Accepted for publication November 6, 2008.

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[1] This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology.

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[3] Abbreviations used in this paper: PSA, passive systemic anaphylaxis; BMMC, bone marrow-derived mast cell; HSA, human serum albumin; HA, hemagglutinin.

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Molecular and Cellular Endocrinology 300 (2009) 32–36

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Characterization of mice deficient in Melanocortin 2 receptor on a B6/Balbc mix background

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ARTICLE INFO

Article history:
Received 27 August 2008
Received in revised form 15 October 2008
Accepted 17 October 2008

Keywords:
Proopiomelanocortin (POMC)
Glucocorticoid (GC)
Melanocortin 2 receptor (MC2R)
Hypothalamic–pituitary–adrenal (HPA) axis
Familial glucocorticoid deficiency (FGD)
Renin–angiotensin–aldosterone system (RAAS)

ABSTRACT

We have previously reported that Melanocortin 2 receptor ($MC2R^{-/-}$) deficient mice on B6 N5 generations exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF) and lack of detectable levels of corticosterone, and reduced serum concentrations of aldosterone and epinephrine. All $MC2R^{-/-}$ mice on B6/N8 background die within 2 days after birth, while about half of the $MC2R^{-/-}$ mice on B6/Balbc mix background survived to adulthood. Both male and female $MC2R^{-/-}$ mice were fertile, suggesting that normal development and function of reproductive organs. $MC2R^{-/-}$ mice delivered from $MC2R^{-/-}$ dams failed to survive due to lung failure, suggesting that fetal or maternal corticosterone is essential for lung maturation. $MC2R^{-/-}$ mice failed to activate the hypothalamic–pituitary–adrenal axis in response to both immune and non-immune stimuli. $MC2R^{-/-}$ mice maintained glomerular structure and achieved electrolyte homeostasis by the activation of the renin–angiotensin–aldosterone system under low aldosterone and undetectable levels of corticosterone.
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1. Introduction

The body responds to stress by activation of the hypothalamic–pituitary–adrenal (HPA) axis and release of glucocorticoids (GCs) under the control of ACTH. ACTH secreted from the anterior pituitary is in turn regulated by hypothalamic corticotropin-releasing hormone (CRH) and Arginine vasopressin (AVP). This HPA axis is regulated by negative feedback exerted by serum cortisol levels on both the hypothalamus and the pituitary gland. ACTH is also the main regulator of adrenal cortical growth. Tissue specific post-translational cleavage of the prohormone, proopiomelanocortin (POMC) gives rise to bioactive peptides including melanotropic peptides, ACTH and several endorphins. We have previously generated mice with an inactivation mutation of the Melanocortin 2 receptor (MC2R) gene, and reported that MC2R on fifth generation of backcrossing to C57/BL6 (B6/N5) leads to neonatal lethality in about three-quarters of $MC2R^{-/-}$ pups, possibly due to hypoglycemia (Chida et al., 2007). Those surviving to adulthood exhibited macroscopically detectable adrenal glands with markedly atrophied zona fasciculata (zF), lack of detectable levels of GC, and reduced serum concentrations of aldosterone and epinephrine (Chida et al., 2007).

GCs are secreted into the systemic circulation from the adrenal cortex and initiate a broad range of actions throughout the organism that regulate the function of multiple organ systems including the central nervous, endocrine, and immune systems. The physiological effects of GCs are mediated by intracellular glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) that function as ligand-dependent transcription factors (Mangelsdorf et al., 1995). Even though MR has a high affinity for GCs, the majority of the physiological effects of GCs are thought to be mediated via the GR, which is expressed more ubiquitously and is a stronger transcriptional activator. Physiologically, MR is thought to act primarily as a high affinity receptor for mineralocorticoids to control the sodium/potassium balance in the kidney and large intestine.

$MC2R^{-/-}$ mice are valuable for familial GC deficiency studies, and a unique animal model for investigation of the physiological functions of GC. The physiological role of GR and MR in vivo have been extensively studied in conditional KO mice (Tronche et al., 1999; Berger et al., 2006), due to the neonatal death of

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doi:10.1016/j.mce.2008.10.027

ELSEVIER

Experimental Hematology

Experimental Hematology 2008;36:897–906

Growth and maturation of megakaryocytes is regulated by Lnk/Sh2b3 adaptor protein through crosstalk between cytokine- and integrin-mediated signals

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(Received 4 December 2007; revised 30 January 2008; accepted 7 February 2008)

Objective. **Various cytokines and growth factors control the differentiation and maturation of megakaryocytes (MKs). However, the mechanism regulating platelet release from MKs is not well understood. Here, we investigated a role of Lnk/Sh2b3, an intracellular adaptor protein, in megakaryopoiesis.**

Materials and Methods. **Number of MK progenitor in bone marrow (BM) of wild-type or *Lnk*$^{-/-}$ mice and their sensitivity to thrombopoietin (TPO) were determined in colony-forming unit assay. Using BM-derived wild-type or *Lnk*$^{-/-}$ MKs stimulated with TPO, activation of the signaling molecules was biochemically analyzed and effect of integrin stimulation on TPO signals was studied by addition of vascular cell adhesion molecule (VCAM-1). Platelet production from MKs in the presence of VCAM-1 was counted by flow cytometry and their morphological change was observed by time-lapse microscopy.**

Results. ***Lnk*$^{-/-}$ mice showed elevated platelets and mature MKs due to enhanced sensitivity of progenitors to TPO. Erkl/2 phosphorylation induced by TPO was augmented and prolonged in *Lnk*$^{-/-}$ MKs while activation of signal transducers and activators of transcription (Stat)3, Stat5, and Akt was normal. Wild-type MKs, but not in *Lnk*$^{-/-}$ MKs on VCAM-1 showed reduced Stat5 phosphorylation and mitogen-activated protein kinases activation upon stimulation with TPO. Additionally, the presence of VCAM in culture accelerated spontaneous platelet release from mature wild-type MKs, but not from *Lnk*$^{-/-}$ MKs.**

Conclusions. **Results suggest that contact of MKs with adhesion molecules via integrins might contribute to platelet release, which is under Lnk-mediated regulation of Stat-5 activation and show that Lnk functions in responses controlled by cell adhesion and in crosstalk between integrin- and cytokine-mediated signaling.** **© 2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Megakaryocytes (MKs) and the platelets they produce are required for normal thrombosis and hemostasis [1]. Recent evidence indicates early roles of platelets in innate immune responses and tumor cell biology [2]. MK progenitors that reside in the bone marrow (BM) undergo endomitosis and differentiate into large, MKs with polyploidy in preparation for platelet production [3]. Thousands of platelets can be released from a single MK into the bloodstream. Thrombopoietin (TPO) is required for both MK development and maintenance of platelet production. Mutant mice lacking TPO or its receptor, c-Mpl, show severe thrombocytopenia [4,5]. However, the remaining platelets in those mice are morphologically and functionally normal [6]. Thus, the TPO/c-Mpl system plays a critical role in the survival and proliferation of MKs, but is not indispensable for either MK maturation or release of platelets.

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doi: 10.1016/j.exphem.2008.02.004

Mod Rheumatol
DOI 10.1007/s10165-009-0154-4

ORIGINAL ARTICLE

Time of initial appearance of renal symptoms in the course of systemic lupus erythematosus as a prognostic factor for lupus nephritis

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Received: 31 October 2008 / Accepted: 19 January 2009
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Abstract The prognosis of lupus nephritis (LN) was studied retrospectively in two LN categories, LN manifested initially at systemic lupus erythematosus (SLE) onset (I-LN) and LN of delayed manifestation after SLE onset (D-LN), based on a chart review (C) of 154 SLE (85 LN) patients with a mean observation of 20.8 ± 9.3 years and a questionnaire study (Q) of 125 LN patients outside our hospital with mean observation of 17.6 ± 9.2 years. In both study groups, half of I-LN patients were relapse-free by Kaplan–Meier analysis after initial therapy, and the relapsed I-LN patients responded to retherapy at higher 5-year relapse-free rates than those of patients receiving initial therapies for D-LN. At last observation, a higher frequency of prolonged remission was shown in I-LN compared with D-LN patients (C: 22/31, 71% versus 14/49, 29%, $P < 0.01$; Q: 65/89, 73% versus 11/33, 33% $P < 0.01$) and also a higher frequency of irreversible renal damage in D-LN compared with I-LN patients (C: 25/49, 51% versus 2/31, 6%, $P < 0.001$; Q: 14/33, 42% versus 6/89, 7%, $P < 0.001$), although class IV pathology was common in patients (C) in both LN categories. Onset time of lupus nephritis in the course of SLE may affect renal prognosis.

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Keywords Lupus nephritis · Onset · Prognosis · SLE

Introduction

Systemic lupus erythematosus (SLE) is a multiple-organ disease, and lupus nephritis (LN) is a major clinical problem because of its high morbidity and mortality rates. In accordance with the chronic nature of SLE, renal symptoms can manifest at various times in the disease course, and a physician cannot predict the future development of LN at the time of SLE onset. Furthermore, it is unclear whether there is a difference in prognosis between LN manifested at the onset of SLE and LN developed later in the course of SLE, because the clinical significance of time of LN onset in the disease course of SLE has not been clearly described in the literature, to our knowledge. Accordingly, in recent clinical trials on therapies for LN including cyclophosphamide and mycophenolate mofetil, mixtures of cases having various time intervals between SLE onset and LN onset have been studied [1, 2]. Although a prognostic impact of renal pathology and a poor prognosis of class IV disease have been established in LN based on the World Health Organization (WHO) classification [3–5] and more recent criteria [3, 6, 7], a later progression or transformation of the pathology cannot necessarily be predicted at the time of the initial biopsy [8, 9].

In our preliminary chart review, we found that numerous cases of remitted SLE had class IV LN at SLE onset. On the other hand, irreversible renal damage was precipitated in patients who initially showed no renal symptoms but later developed LN with various renal pathologies, and the LN of later development was never a case of senile-onset LN. Thus we undertook to study the possible relationship

Published online: 10 March 2009

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Molecular Immunology 44 (2007) 2638–2646

Molecular Immunology

www.elsevier.com/locate/molimm

Recognition of H-2K^b by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function☆

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Received 10 November 2006; received in revised form 12 December 2006; accepted 13 December 2006
Available online 22 January 2007

Abstract

Ly49Q is a member of the polymorphic Ly49 family of NK cell receptors that displays both a high degree of conservation and a unique expression pattern restricted to myeloid lineage cells, including plasmacytoid dendritic cells (pDC). The function and ligand specificity of Ly49Q are unknown. Here, we use reporter cell analysis to demonstrate that a high-affinity ligand for Ly49Q is present on H-2^b, but not H-2^d, H-2^k, H-2^q, or H-2^a-derived tumor cells and normal cells *ex vivo*. The ligand is peptide-dependent and MHC Ia-like, as revealed by its functional absence on cells deficient in TAP-1, β_2m, or H-2K^{b}D^b expression. Furthermore, Ly49Q is specific for H-2K^b, as the receptor binds peptide-loaded H-2K^b but not H-2D^b complexes, and Ly49Q recognition can be blocked using anti-K^b but not anti-D^b mAb. Greater soluble H-2K^b binding to ligand-deficient pDC also suggests *cis* interactions of Ly49Q and H-2K^b. These results demonstrate that Ly49Q efficiently binds H-2K^b ligand, and suggest that pDC function, like that of NK cells, is regulated by classical MHC Ia molecules. MHC recognition capability by pDC has important implications for the role of this cell type during innate immune responses.
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Keywords: Plasmacytoid dendritic cells; MHC; Ly49Q; H-2K^b; Ligand-identification

1. Introduction

Plasmacytoid dendritic cells (pDC), also known as interferon-producing cells (IPC), are a specialized subset of DC especially suited for initiating immune responses to viruses. Production of abundant IFN-α levels as well as IL-12 and other cytokines by pDC has direct anti-viral effects on infected cells, and further activates effector cells such as natural killer (NK) cells to destroy infected targets. Plasmacytoid DC express TLR7 and TLR9, allowing for recognition of viral single-stranded RNA and unmethylated CpG-containing double-stranded DNA, respectively, in endosomal compartments (Liu, 2005). Likewise, expression of TLR9 by pDC and myeloid dendritic cells (mDC) is necessary for protection against mouse cytomegalovirus (MCMV) challenge (Krug et al., 2004; Tabeta et al., 2004). In addition to activating NK cells via type I interferon, pDC also augment CD8$^+$ T cell cytotoxicity (Salio et al., 2004). Furthermore, activated pDC upregulate T cell costimulatory molecules such as CD86 and class II MHC molecules, allowing them to present antigenic peptides and stimulate CD4$^+$ T cell function (Nakano et al., 2001). In turn, pDC have been

Abbreviations: B6, C57BL/6; NK, natural killer; DC, dendritic cells; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cells; MHC, major histocompatibility complex

☆ Animal care was provided in accordance with the procedures approved by the IRCM Animal Care Committee.

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0161-5890/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2006.12.010

Inhibition of CCL1-CCR8 Interaction Prevents Aggregation of Macrophages and Development of Peritoneal Adhesions[1]

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Peritoneal adhesions are a significant complication of surgery and visceral inflammation; however, the mechanism has not been fully elucidated. The aim of this study was to clarify the mechanism of peritoneal adhesions by focusing on the cell trafficking and immune system in the peritoneal cavity. We investigated the specific recruitment of peritoneal macrophages (PMϕ) and their expression of chemokine receptors in murine models of postoperative and postinflammatory peritoneal adhesions. PMϕ aggregated at the site of injured peritoneum in these murine models of peritoneal adhesions. The chemokine receptor CCR8 was up-regulated in the aggregating PMϕ when compared with naive PMϕ. The up-regulation of CCR8 was also observed in PMϕ, but not in bone marrow-derived Mϕ, treated with inflammatory stimulants including bacterial components and cytokines. Importantly, CCL1, the ligand for CCR8, a product of both PMϕ and peritoneal mesothelial cells (PMCs) following inflammatory stimulation, was a potent enhancer of CCR8 expression. Cell aggregation involving PMϕ and PMCs was induced in vitro in the presence of CCL1. CCL1 also up-regulated mRNA levels of plasminogen activator inhibitor-1 in both PMϕ and PMCs. *CCR8* gene-deficient mice or mice treated with anti-CCL1-neutralizing Ab exhibited significantly reduced postoperational peritoneal adhesion. Our study now establishes a unique autocrine activation system in PMϕ and the mechanism for recruitment of PMϕ together with PMCs via CCL1/CCR8, as immune responses of peritoneal cavity, which triggers peritoneal adhesions. ***The Journal of Immunology,*** **2007, 178: 5296–5304.**

The serosal membrane of viscera and the peritoneal cavity are involved in numerous types of inflammation and surgical intervention. For example, in the case of surgery, postoperative adhesions occur in the majority of patients following laparotomy and laparoscopy (1, 2). Peritoneal adhesions cause significant signs and symptoms including intestinal obstruction, chronic pelvic pain and infertility, and eventually a second more serious surgery is often required. Thus, adhesions in the peritoneal cavity are both life-threatening and an enormous cost for patient care. For example, 34.6% of patients who had undergone intra-abdominal surgery were readmitted within the next 10 years for a disorder directly or possibly related to adhesions, or for abdominal or pelvic surgery that could be potentially complicated by adhesions (2). Despite the large number of surgical operations performed daily, the mechanism for peritoneal adhesions is not well-understood. Previous reports showed that peritoneal injury is triggered by leakage of plasma proteins, followed by formation of fibrinous deposits and proliferation of fibroblasts (3). A rapid and transient influx of neutrophils into the peritoneal cavity also occurs followed by an accumulation of mononuclear cells, largely macrophages (Mϕ)[3] (4, 5). CD4-positive T cells also play a significant role in peritoneal adhesions together with the T cell-derived proinflammatory cytokine, IL-17 (6), and the programmed death-1 inhibitory pathway (7). Although active roles for these cells in adhesions have been shown (8, 9), little is yet known about the cell origin or the dynamics of migration to help explain the peritoneal adhesion events. Inflammation such as appendicitis, endometriosis, and pelvic inflammatory disease can also cause peritoneal adhesion, which can lead to infertility and reproductive problems. In the case of Crohn's disease, intestinal transmural ulcerations with fissures or fistulas are the most important pathological findings (10). These Crohn's disease lesions involve the intestinal serosa and mesentery. The characteristic changes in the serosal surface, including fat wrapping, correlate directly with overall extent of inflammatory changes: the stricture of the intestine (10, 11), the depth of lymphoid aggregate penetration, and the number of lymphoid aggregates in the underlying ileal wall (12). These observations suggest that inflammation of viscera is not limited to the organ, but provokes responses in the peritoneal cavity as well. Most importantly, pathological changes in the peritoneal cavity cause serious symptoms and directly affect the quality of life of patients.

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Received for publication October 17, 2006. Accepted for publication January 23, 2007.

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[1] This work was supported in part by Medical Techniques Promotion Research Grant H14-nano-004 from the Ministry of Health, Labor, and Welfare of Japan; grants and contracts from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Japan Health Sciences Foundation; and the Japan Science and Technology Agency.

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[3] Abbreviations used in this paper: Mϕ, macrophage; BMϕ, bone marrow-derived Mϕ; PMϕ, peritoneal Mϕ; QD, quantum dot; PGN, peptidoglycan; pAb, polyclonal Ab; TNBS, 2,4,6-trinitrobenzene sulfonic acid; PTX, pertussis toxin; CIMA, chemokine-induced macrophage aggregation; PMC, peritoneal mesothelial cell; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

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Dose-Dependent Differential Regulation of Cytokine Secretion from Macrophages by Fractalkine[1]

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Although expression of the fractalkine (CX3CL1, FKN) is enhanced in inflamed tissues, it is detected at steady state in various organs such as the intestine, and its receptor CX3CR1 is highly expressed in resident-type dendritic cells and macrophages. We hypothesized that FKN might regulate the inflammatory responses of these cells. Therefore, murine macrophages were pretreated with FKN and then stimulated with LPS. We found that macrophages pretreated with 0.03 nM FKN but not with 3 nM FKN secreted 50% less TNF-α than did cells treated with LPS alone. Cells treated with 0.03 nM FKN and LPS also showed reduced phosphorylation of ERK1/2 and reduced NF-κB p50 subunit. Interestingly, the p65 subunit of NF-κB was translocated to the nuclei but redistributed to the cytoplasm in the early phase by forming a complex with peroxisome proliferator-activated receptor (PPAR) γ. Exogenous 15-deoxy-Δ(12,14)-prostaglandin J2, a natural ligand for PPAR-γ, also induced redistribution of p65 with decreased TNF-α secretion after LPS challenge. Pretreatment with 0.03 nM but not 3 nM FKN increased the cellular levels of 15-deoxy-Δ(12,14)-prostaglandin J2 as well as mRNA of PPAR-γ. Requirement of PPAR-γ for the effect of 0.03 nM FKN was confirmed by small interfering RNA of PPAR-γ. In contrast, pretreatment with 3 nM FKN induced higher levels of IL-23 compared with cells pretreated with 0.03 nM FKN and produced TNF-α in a CX3CR1-dependent manner. These dose-dependent differential effects of FKN establish its novel role in immune homeostasis and inflammation. *The Journal of Immunology,* 2007, 179: 7478–7487.

Fractalkine (CX3CL1, FKN[3]) is a unique chemokine produced as a membrane-bound molecule that consists of an intracellular tail, a short membrane-spanning region, and a glycosylated mucin-like stalk that extends from the cell surface holding the chemokine domain (1). FKN also exists as a soluble glycoprotein that is produced by proteolytic cleavage of the full-length molecule at a membrane-proximal site (2, 3). Expression of FKN in endothelial cells is induced by various inflammatory stimuli such as LPS, TNF-α, IL-1, and IFN-γ (4–6). Besides induction of chemotaxis, FKN also functions as an adhesion molecule to support leukocyte adhesion and transmigration (7, 8). A unique receptor for FKN, CX3CR1, is expressed abundantly by dendritic cells and macrophages/monocytes (9–11) as well as Th-type 1 cells, cytotoxic effector lymphocytes (12, 13), mast cells (14), neurons, astrocytes, and microglia (15–17). Since expression of FKN and expression of CX3CR1 can be induced by immune cells, studies have focused on the role of FKN as an inflammatory mediator. Indeed, FKN is up-regulated in the inflammatory site of rheumatoid arthritis (12, 18), inflammatory bowel disease (19), atherosclerosis (20), psoriasis (21), myositis (22), and various inflammatory conditions of the kidney (23) and brain (24), although FKN gene-disrupted mice did not show significant differences from wild-type mice in either steady-state or inflammatory conditions (25).

Conversely, studies using GFP/CX3CR1 knock-in mice have shown that a CX3CR1^{high}CCR2^{-}Gr1^{-} subset of murine blood monocytes characterized by CX3CR1-dependent recruitment to noninflamed tissues and a short-lived CX3CR1^{low}CCR2^{+}Gr1^{+} cell population is actively recruited to inflamed tissue (26). Furthermore, CX3CR1-positive dendritic cells are distributed abundantly in the lamina propria of the normal intestine (27). Recent studies have shown that circulating CX3CR1^{+}CD117Lin^{-} precursors represent the origin of some subsets of resident macrophages and dendritic cells (28), and a small proportion of intestinal lymph dendritic cells are derived from CX3CR1^{high} blood monocytes in vivo under steady-state conditions (29). These results indicated the role of the FKN/CX3CR1 system in homing of noninflammatory or resident subsets of dendritic cells and macrophages. Of interest, a considerable amount of FKN is produced by epithelial cells and other types of cells in the normal intestine (19, 30). In addition to its role in cell dynamics, we assumed that the physiological level of FKN in the intestine regulates the function of CX3CR1^{+} macrophages. Resident macrophages in the normal intestine have the distinctive feature of hyporesponsiveness to various inflammatory stimuli, including bacterial components (31–33). This is in sharp contrast to circulating monocytes and splenic macrophages, which

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Received for publication January 9, 2007. Accepted for publication September 25, 2007.

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[1] This work was supported in part by grants and contracts from the Ministry of Health, Labour and Welfare, the Ministry of Education, Culture, Sports, Science and Technology, the Japan Health Sciences Foundation, Novartis Foundation (Japan) for the Promotion of Science, and the Mitsukoshi Health and Welfare Foundation.

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[3] Abbreviations used in this paper: FKN, fractalkine (CX3CL1); BMϕ, bone marrow-derived macrophage; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; PPAR-γ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

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Ly49B Is Expressed on Multiple Subpopulations of Myeloid Cells[1]

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Using a novel mAb specific for mouse Ly49B, we report here that Ly49B, the last remaining member of the C57 Ly49 family to be characterized, is expressed at low levels on ~1.5% of spleen cells, none which are NK cells or T cells but which instead belong to several distinct subpopulations of myeloid cells defined by expression of CD11b and different levels of Gr1. Much larger proportions of bone marrow and peritoneal cells expressed Ly49B, all being CD11b$^+$ and comprising multiple subpopulations defined by light scatter, F4/80, and Gr1 expression. Costaining for Ly49Q, also expressed on myeloid cells, revealed that Ly49B and Ly49Q were most strongly expressed on nonoverlapping subpopulations, Ly49Q^{high} cells being mostly B220$^+$CD4$^+$ and/or CD8$^+$, Ly49B$^+$ cells lacking these markers. Myeloid populations that developed from bone marrow progenitors in vitro frequently coexpressed both Ly49B and Ly49Q, and Ly49B expression could be up-regulated by LPS, α-IFN, and γ-IFN, often independently of Ly49Q. PCR analysis revealed that cultured NK cells and T cells contained Ly49B transcripts, and Ly49B expression could be detected on NK cells cultured in IL-12 plus IL-18, and on an immature NK cell line. Immunohistochemical studies showed that Ly49B expression in tissues overlapped with but was distinct from that of all other myeloid molecules examined, being particularly prominent in the lamina propria and dome of Peyer's patches, implicating an important role of Ly49B in gut immunobiology. In transfected cells, Ly49B was found to associate with SHP-1, SHP-2, and SHIP in a manner strongly regulated by intracellular phosphorylation events. ***The Journal of Immunology,*** **2006, 177: 5840–5851.**

Since their discovery in 1983 (1), Ly49 receptors have been the subject of intense interest. It was eventually realized that they are encoded by a series of closely related genes (2) clustered at the telomeric end of the NK gene complex, a region of ~2 Mb on chromosome 6 that contains several families of genes expressed predominantly in NK cells (3–5). The Ly49 polypeptides are type II transmembrane proteins belonging to the C-type lectin superfamily and comprise two subclasses, one of which possesses an ITIM in the cytoplasmic domain and delivers inhibitory signals following cross-linking by MHC class I ligands, while the other lacks an ITIM, has a charged residue in the transmembrane domain, and delivers activatory signals via the adaptor protein DAP12 following interaction with ligands that may include MHC class I molecules and virus-encoded class I homologs (5). Most of the Ly49 receptors that have been studied are expressed predominantly on NK cells, although the inhibitory Ly49s can also be expressed in some circumstances on T cells (6). The only member of the Ly49 family in C57 mice that has so far not been studied is Ly49B (2). The gene encoding Ly49B is separated by >0.8 Mb from the cluster containing the remaining 10 functional Ly49 genes (4). It has an intron-exon structure similar to that of other Ly49 genes but appears to lack the upstream Pro1 element responsible for initiating the stochastic expression of Ly49 receptors in NK cells (7). Ly49B cDNAs have been obtained from several tissue sources and predict a polypeptide that lacks a transmembrane charge and bears an ITIM in the cytoplasmic domain but which is significantly divergent from other Ly49s. Interestingly, Ly49B is the only member of the mouse Ly49 family that has an identifiable ortholog in the rat (8, 9), suggesting it may have a distinctive and conserved function in murine species. We have now generated a series of mAbs that specifically recognize Ly49B and report here that Ly49B is not normally expressed on NK cells but instead is expressed on multiple subpopulations of myeloid cells that include granulocytes and macrophages.

Materials and Methods

Cells

PVG female rats and C57BL/6 and BALB/c female mice were obtained from Harlan-Olac, and all procedures were approved by the U.K. licensing authorities. Except where stated, 6- to 16-wk-old C57BL/6 mice were used as cell donors. Spleen cells were obtained by teasing of spleens with forceps, bone marrow (BM)[3] cells by passing material eluted from femurs and tibias through narrow gauge needles, and peritoneal cells by flushing the peritoneum with PBS. In some experiments, animals were injected i.p. with 1 ml of a 10% solution of thioglycollate broth.

Cultures

The culture medium used was DMEM (52100-039; Invitrogen Life Technologies) made up in highly purified water and supplemented with 2× nonessential amino acids, 5×10^{-5} M 2-ME, and 10% FBS (F-7524; Sigma-Aldrich). Spleen and BM macrophages were obtained by culturing aliquots of respectively 50 or 5 million nucleated cells in 90-mm bacteriological grade petri dishes containing 10 ml of medium supplemented with 10% heat-inactivated horse serum and 10% supernatant from L929 cells

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Received for publication January 11, 2006. Accepted for publication July 25, 2006.

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[1] This work was generously supported by grants from the Biotechnology and Biological Sciences Research Council, U.K.

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[3] Abbreviations used in this paper: BM, bone marrow; cII, MHC class II; CRD, carbohydrate recognition domain; DC, dendritic cell; FL, flt3 ligand; HA, hemagglutinin; SHP, Src homology region 2 domain-containing phosphatase; SSC, side light scatter.

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0022-1767/06/$02.00

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Rac1-mediated Bcl-2 induction is critical in antigen-induced CD4 single-positive differentiation of a CD4$^+$CD8$^+$ immature thymocyte line

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Abstract: Rac1, one of the Rho family small guanosine triphosphatases, has been shown to work as a "molecular switch" in various signal transduction pathways. To assess the function of Rac1 in the differentiation process of CD4 single-positive (CD4-SP) T cells from CD4CD8 double-positive (DP) cells, we used a DP cell line DPK, which can differentiate into CD4-SP cells upon TCR stimulation in vitro. DPK expressing dominant-negative (dn)Rac1 underwent massive apoptosis upon TCR stimulation and resulted in defective differentiation of CD4-SP cells. Conversely, overexpression of dnRac2 did not affect differentiation. TCR-dependent actin polymerization was inhibited, whereas early ERK activation was unaltered in dnRac1-expressing DPK. We found that TCR-dependent induction of Bcl-2 was suppressed greatly in dnRac1-expressing DPK, and this suppression was independent of actin rearrangement. Furthermore, introduction of exogenous Bcl-2 inhibited TCR-dependent induction of apoptosis and restored CD4-SP generation in dnRac1-expressing DPK without restoring TCR-induced actin polymerization. Collectively, these data indicate that Rac1 is critical in differentiation of CD4-SP from the DP cell line by preventing TCR-induced apoptosis via Bcl-2 up-regulation. *J. Leukoc. Biol.* 81: 500–508; 2007.

Key Words: *T lymphocytes · TCRs · apoptosis · thymus*

INTRODUCTION

Rac1 belongs to the Rho family of small guanosine triphosphatases (GTPases), which play critical roles in actin cytoskeletal rearrangement in many cell systems. Among the Rho family GTPases, Rac1 has a broad range of guanine nucleotide exchanging factors (GEFs) and effectors so that the molecule acts as a molecular switch in many aspects of signal transduction pathways. Recent studies using transgenic technology have revealed that Rho family GTPases play crucial roles in thymocyte development and TCR-mediated signal transduction [1]. Ectopic expression of bacterial C3T, which inhibits RhoA, -B, and -C, resulted in decreased numbers of CD4CD8 double-positive (DP) cells in the thymus [2], and transgenic expression of constitutively active RhoA resulted in enhanced positive selection [3]. Constitutively active cdc42 induced massive apoptosis in DP thymocytes [4], suggesting that cdc42 is also involved in T cell development.

Rac consists of three independent genes: Rac1, -2, and -3. Rac1 is expressed ubiquitously, whereas expression of Rac2 is restricted to hematopoietic cells. Rac2-deficient mice showed normal T cell development in the thymus, defective Th1 differentiation caused by decreased IFN-γ production [5], perturbed chemotaxis [6], and defective T cell activation accompanied by decreased ERK activation [7]. Rac2, a component of NADPH oxidase, plays a critical role in reactive oxygen species in phagocytes [8], and recently, Rac1 was shown to play a similar role in human macrophages [9]. Transgenic expression of constitutively active Rac1 (L61) generates DP thymocytes in a RAG$^{-/-}$ background [10] and converts positive selection to negative selection [11], indicating that Rac1 regulates the strength of TCR-mediated signal transduction. Rac1-deficient mice are embryonic-lethal, and neutrophil-specific disruption of Rac1 was reported [12]. Recently, conditional knockout mice for Rac1 in a Rac2$^{-/-}$ background were generated, and differential, critical roles of Rac1 and Rac2 in growth and engraftment of hematopoietic stem cells [13–15] as well as in B cell development [16] were reported. However, the effect of each Rac in T cell development is still unknown. As all three Racs are expressed in T cells, we have studied the role of Rac in T cell development using a dominant-negative (dn) strategy. Using DPK, a DP thymic lymphoma capable of differentiation into CD4 single-positive (SP) cells upon antigenic stimulation in vitro [17], we demonstrate that activation of Rac1 is required

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Received October 17, 2005; revised September 20, 2006; accepted October 5, 2006.

doi: 10.1189/jlb.1005585

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The p85α Regulatory Subunit of Class IA Phosphoinositide 3-Kinase Regulates β-Selection in Thymocyte Development[1]

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We examined the role of class IA PI3K in pre-TCR controlled β-selection and TCR-controlled positive/negative selection in thymic development. Using mice deficient for p85α, a major regulatory subunit of the class IA PI3K family, the role of class IA PI3K in β-selection was examined by injection of anti-CD3ε mAb into p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice, which mimics pre-TCR signals. Transition of CD4$^-$CD8$^-$ double-negative (DN) to CD4$^+$CD8$^+$ double-positive (DP) thymocytes triggered by anti-CD3ε mAb was significantly impaired in p85$\alpha^{-/-}$Rag-2$^{-/-}$ compared with p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice. Furthermore, DP cell numbers were lower in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ TCR-transgenic mice than in DO11.10/Rag-2$^{-/-}$ mice. In addition, inhibition by IC87114 of the major class IA PI3K catalytic subunit expressed in lymphocytes, p110δ, blocked transition of DN to DP cells in embryonic day 14.5 fetal thymic organ culture without affecting cell viability. In the absence of phosphatase and tensin homolog deleted on chromosome 10, where class IA PI3K signals would be amplified, the DN to DP transition was accelerated. In contrast, neither positive nor negative selection in Rag-2$^{-/-}$TCR-transgenic mice was perturbed by the lack of p85α. These findings establish an important function of class IA PI3K in the pre-TCR-controlled developmental transition of DN to DP thymocytes. ***The Journal of Immunology,*** **2007, 178: 1349–1356.**

T cell development in the thymus is a highly controlled process beginning with the most immature thymocyte, termed CD4$^-$CD8$^-$ double-negative (DN)[3] (1–3). The DN progenitors can be subdivided into four different stages based on their CD44 and CD25 expression patterns. The earliest progenitors are CD44$^+$CD25$^-$ (DN1) cells, followed by CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$ (DN2–4, respectively) cells. After successful rearrangement of the TCRβ, the CD44$^-$CD25$^+$ (DN3) cells express a pre-TCR on their surface made up of the TCRβ protein and a pTα. After passing the first T cell developmental checkpoint, namely β-selection, DN3 cells make the transition to the CD4$^+$CD8$^+$ double-positive (DP) stage. This transition includes cell proliferation triggered by signals through the pre-TCR. The pre-TCR also triggers rearrangement of the *TCRα* gene, leading to the expression of TCR$\alpha\beta$ heterodimers on DP cells. These cells then go through a second checkpoint, termed positive and negative selection. DP cells receiving weak signals through the TCR survive and differentiate into mature CD4$^+$ or CD8$^+$ single-positive (SP) cells (positive selection), whereas those receiving strong signals are eliminated (negative selection). Furthermore, those incapable of recognizing appropriate MHC molecules fail to receive the TCR signal and are also eliminated (death by neglect) (1–3).

PI3Ks are lipid kinases that specifically phosphorylate the D3 position of the inositol ring of phosphatidylinositol (PI) species (reviewed in Refs. 4–7). PI3Ks are activated through a variety of extracellular stimuli and promote assembly of signaling complexes at the plasma membrane. PI(3,4)P$_2$ and PI(3,4,5)P$_3$, products of PI3Ks, recruit specific signaling proteins containing a pleckstrin homology domain that selectively binds 3-phosphoinositides. These signaling proteins include phosphoinositide-dependent kinase 1 (PDK1), Akt, and Vav1 and are involved in a wide range of cellular processes such as cell metabolism, cell cycle progression and survival. PI3K-signaling pathways are counteracted by phosphatase and tensin homologue deleted on chromosome 10 (Pten), a 3-phosphoinositide-specific lipid phosphatase.

The class IA PI3Ks are heterodimeric proteins composed of a catalytic (p110α, p110β, or p110δ) and a regulatory (p85α, p55α, p50α, p85β, or p55γ) subunit and are activated mostly downstream of protein tyrosine kinases (4–7). Each regulatory subunit can interchangeably associate with different catalytic subunits and shows a unique tissue distribution. p85α, a product encoded by the *Pik3r1* gene, is the major regulatory subunit of class IA PI3Ks in most types of cells including immune cells. The *Pik3r1* gene encodes two alternative splicing forms in addition to p85α, p55α, and p50α. Mice lacking p85α alone or mice lacking all products encoded by the *Pik3r1* gene (p85α, p55α, and p50α) show comparable immune phenotypes such that B cell development and activation are impaired while T cell functions are apparently unaffected (8–10). In contrast, mice lacking the p85β regulatory subunit or transgenic (tg) mice

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Received for publication July 12, 2006. Accepted for publication November 16, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Mitsubishi Foundation, a Keio University Special Grant-in-Aid for Innovative Collaborative Research Project, a Grant-in-Aid for Scientific Research for Young Scientist (16790293 to S.M.) from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research on Priority Areas (14021110 to S.K. and 16043248 to S.M.), a National Grant-in-Aid for the Establishment of a High-Tech Research Center in a private university, and a Scientific Frontier Research Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; tg, transgenic; PI, phosphatidylinositol; Pten, phosphatase and tensin homolog deleted on chromosome 10; PDK, phosphoinositide-dependent kinase; 7AAD, 7-aminoactinomycin D; FTOC, fetal thymic organ culture.

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