12. Spores from wild-type or Ura$^+$ heterozygous diploid (*11*) were germinated in EMM2 at 33°C for 16 hours with and without uracil. Disruptant spores carrying the integrated *ura4*$^+$ gene could germinate in the absence of uracil. MNase digestion was performed as described (*2*).
13. A L87Q mutation similar to *cse4-103* was introduced into the SpCENP-A gene with the native promoter. The resultant *cnp1-1* mutant gene was integrated at the *lys1* locus of SpCENP-A–null strain.
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24. *cdc25-22 ts* mutant cells [P. A. Fantes, *Nature* **279**, 428 (1979)] were shifted to 36°C for the arrest in G_2. Cells were synchronously released into mitosis by the shift to 26°C.
25. We thank M. M. Smith for *cse4-103* mutant and A. M. Carr for the gift of the *S. pombe* genomic DNA library. The present study was supported by the CREST research project of Japan Science and Technology Corporation.

25 February 2000; accepted 24 April 2000

Role of the Guanosine Triphosphatase Rac2 in T Helper 1 Cell Differentiation

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T helper 1 (T_H1) cells mediate cellular immunity, whereas T_H2 cells potentiate antiparasite and humoral immunity. We used a complementary DNA subtraction method, representational display analysis, to show that the small guanosine triphosphatase Rac2 is expressed selectively in murine T_H1 cells. Rac induces the interferon-γ (IFN-γ) promoter through cooperative activation of the nuclear factor kappa B and p38 mitogen-activated protein kinase pathways. Tetracycline-regulated transgenic mice expressing constitutively active Rac2 in T cells exhibited enhanced IFN-γ production. Dominant-negative Rac inhibited IFN-γ production in murine T cells. Moreover, T cells from Rac2$^{-/-}$ mice showed decreased IFN-γ production under T_H1 conditions in vitro. Thus, Rac2 activates T_H1-specific signaling and IFN-γ gene expression.

T_H1 and T_H2 cells can be differentiated in vitro from common naïve precursor T cells during the course of a few days (*1*). During this time period, T_H1 or T_H2 regulatory proteins specific to each lineage are induced that are likely to play key roles in the differentiation process. To search for genes differentially expressed in T_H1 or T_H2 cells, we performed RDA (representational display analysis) (*2*) using in vitro–differentiated T_H1 and T_H2 cells. Using this procedure, we identified the transcription factor GATA3 as a T_H2-specific gene and a key regulator of T_H2 differentiation (*3*). Here we show that the small guanosine 5′-triphosphate (GTP)–binding protein Rac2 is a T_H1-specific gene that plays a central role in T_H1 development. A T_H1 probe generated after three rounds of RDA subtraction with primary T_H1 and T_H2 cDNA was used to screen a T_H1 cDNA library. Rac 2 was one of the genes identified. To confirm this result, we examined the expression level of Rac2 in day 4 T_H1 and T_H2 cells. A highly enriched Rac2 mRNA representation was found in primary T_H1 cells (Fig. 1A) while, as expected, GATA3 showed enrichment in T_H2 cells.

Rac has been shown to activate both JNK, p38, and NF-κB pathways (*4*–*6*) in various cell types. Moreover, JNK and p38 are selectively activated in T_H1 effector cells (*7*, *8*). This suggested that the elevated level of Rac2 might be the cause of the selective activation of these pathways. Consistent with these observations, constitutively active Rac2 (L61, GTP-bound) activated JNK and p38 pathways and NFκB in Jurkat cells, as shown by both direct measurement of JNK and p38 kinase activity (Fig. 1B) and AP1-luciferase (*9*), Chop-luciferase (*10*), and κB-luciferase (*11*) reporter gene activation (Fig. 1C), while having no effect on a reporter construct bearing binding sites for the ets family of transcription factors. A similar result was observed with Rac1L61 (*12*).

We first examined whether Rac2 plays a role in T_H1 cytokine gene expression. Constitutively active Rac2 cotransfected into Jurkat cells with an interferon-γ (IFN-γ) promoter reporter plasmid (*13*) induced a six- to sevenfold activation of the IFN-γ promoter, whereas CDC42L61 did not (Fig. 2A). To determine whether Rac2L61 activates IFN-γ expression in T cell clones, we cotransfected IFN-γ promoter reporter and the expression vector for Rac2L61 into the T_H1 clone AE7 (*14*) and the T_H2 clone D10 (*15*). Although Rac2L61 strongly activated the IFN-γ promoter in AE7 cells, it failed to activate the IFN-γ promoter in D10 cells (Fig. 2B).

To examine which of these signaling pathways—JNK, p38, or NF-κB–is required for Rac-mediated IFN-γ activation, we blocked each pathway with specific inhibitors. Whereas dominant-negative JNK1 or the ERK inhibitor PD98059 has no effect on RacL61-induced IFN-γ promoter activation, both the NF-κB super repressor (*16*) and the p38 inhibitor SB203580 inhibited this activation completely (Fig. 2C). We next examined whether any of these pathways was sufficient for Rac-mediated IFN-γ activation. Whereas activation of the JNK pathway with MKK7 and JNK1 (*17*), the p38 pathway with MKK6glu (*18*), or NF-κB with a constitutively active form of IKKβ (*19*) alone was not sufficient to activate the IFN-γ promoter, activation of both the p38 pathway and NF-κB together induced IFN-γ promoter activity to a level similar to that obtained with RacL61 (Fig. 2D). The JNK pathway does not appear to contribute to IFN-γ promoter activity, because it did not synergize with either NF-κB or p38. Thus, both the p38 and NF-κB pathways are required for Rac-mediated IFN-γ activation, and these two pathways activate the IFN-γ promoter synergistically.

To investigate the role of Rac in T_H1

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differentiation in vivo, we used a reverse tetracycline-controlled transactivator (rtTA)–regulated transgenic mouse system (*20*, *21*) to express Rac2L61 in T cells (*22*). Although a low level of leaky expression was observed in transgene-positive mice before doxycycline induction, T and B lymphocyte development was normal (*12*). Strong induction of Rac2L61 transgene expression was observed after doxycycline treatment (Fig. 3A). No induction was observed in the transgene-negative littermates. Transgene expression was also confirmed by Western blot analysis with a monoclonal antibody against Myc tag (Fig. 3B).

We examined the effect of transgene expression on expression of cytokine genes using a ribonuclease protection assay (Fig. 3C) (*23*). Production of the two T_H1 cytokines IFN-γ and LTα was substantially enhanced in transgenic T cells compared with T cells from negative littermates, whereas lineage-nonspecific cytokines like tumor necrosis factor–α were unaffected. Quantitation by cytokine enzyme-linked immunosorbent assay (ELISA) showed a fourfold increase of IFN-γ in transgenic mice (Fig. 3D). In comparison, interleukin-4 (IL-4) levels were essentially unaffected. To determine whether Rac2L61 transgenic T cells were predisposed to produce IFN-γ in vivo, we immunized doxycycline-treated mice with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant (CFA). Rac2L61 expression increased the level of IFN-γ production by T cells from draining lymph nodes significantly, but had little effect on IL-4 production or T cell proliferation. Thus, Rac activation is sufficient to induce IFN-γ production in T cells in vivo.

To determine whether Rac is necessary for IFN-γ production in T cells, we first expressed the dominant-negative (N17, guanosine diphosphate–bound) mutants of Rac1, which inhibits both Rac1 and Rac2, or CDC42N17 in Jurkat cells. Rac1N17 greatly inhibited phorbol 12-myristate 13-acetate (PMA)–ionomycin–induced IFN-γ promoter activation, whereas CDC42N17 had no significant effect (Fig. 4A). To examine the effect of Rac inactivation on IFN-γ expression in primary T cells, we generated recombinant retrovirus-expressing Rac1N17 under the control of the Moloney leukemia virus long-terminal repeat (MLVLTR). In vitro–stimulated primary CD4 T cells were activated, infected with virus and assayed after 5 days in culture (*24*). Equal proportions of cells positively stained for IFN-γ were observed in the infected and uninfected populations of T cells infected with control green fluorescent protein (GFP) vector (Fig. 4B). In cells infected with virus-expressing RAC1N17, however, IFN-γ was reduced to background levels (Rac1N17-GFP vector).

Finally, we examined whether Rac2 plays a critical role in T_H1 cytokine production under T_H1-polarized conditions using Rac2 knockout mice (*25*). These animals have normal T lymphocyte development (*25*, *26*). Wild-type CD4 T cells develop a strong T_H1 response after primary culture with ConA,

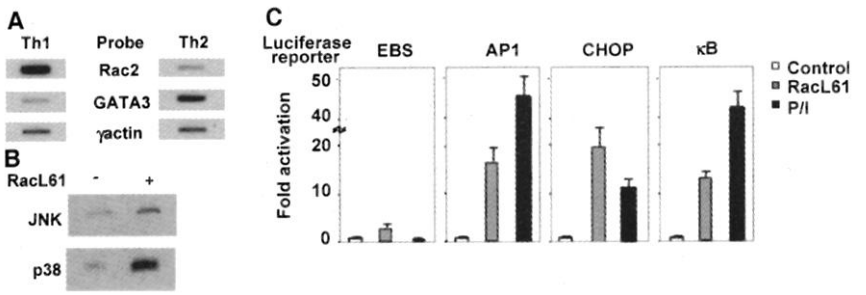

Fig. 1. Identification of Rac2 as a T_H1-specific gene. RDA analysis with cDNA from day 4 cultured T_H1 and T_H2 cells was performed. A T_H1 probe was derived and used to screen a T_H1 library, which was used to identify the Rac2 gene. (**A**) Slot-blot analysis of 50 ng of Rac2, GATA3, and γ-actin hybridized with radioactive labeled day 4 T_H1 and T_H2 initial representation probe. The bands were quantitated with a PhosphorImager (Becton-Dickinson). The Rac2 signal is sevenfold higher in the T_H1 than in the T_H2 sample, and the GATA3 signal is fivefold higher in the T_H2 than in the T_H1 sample. (**B**) The JNK and p38 MAP kinase in Rac2L61-transfected Jurkat cell extract were immunoprecipitated, and the MAP kinase activity was measured in the immune complex by protein kinase assay with recombinant c-Jun and ATF2 as substrate, respectively. (**C**) Jurkat cells were transfected with 3 μg of EBS-luciferase, collagenase AP1-luciferase, Chop-luciferase (Chop expression vector and pFR-luciferase construct), or κB-luciferase reporter plasmids with (shaded bars) or without (open and black bars) RacL61 and 100 ng of PRL-luciferase. PMA-ionomycin was added 4 hours after transfection (black bars). Dual luciferase assay was performed 24 hours after transfection.

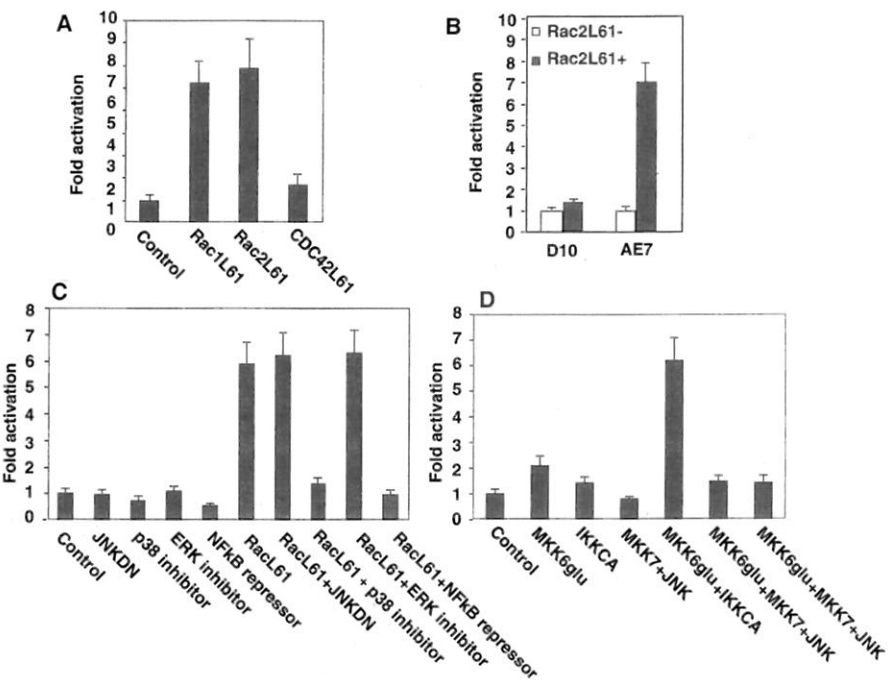

Fig. 2. IFN-γ promoter activation by Rac. Dual luciferase assay in Jurkat cells was performed as described in Fig. 1C (A, B, and D). IFNγluc (3 μg) and expression vectors (6 μg) for active or negative mutants of Rho family small heterotrimeric GTP binding proteins (G proteins) were used. The values of the control samples were normalized as 1. (**A**) Cells were cotransfected with IFNγluc and Rac1L61, Rac2L61, or CDC42L61. (**B**) AE7 (a T_H1 clone) (*9*) or D10 cells (a T_H2 clone) (*9*) were transfected with IFNγluc reporter plasmid with or without the expression vector for Rac2L61. Sixteen hours later, cells were stimulated with APC and antigen for 24 hours, and dual luciferase activity was assayed. (**C**) Jurkat cells were transfected with IFNγluc with or without RacL61 expression vector and either cotransfected with 5 μg of plasmids expressing inhibitors of various signal transduction pathways or treated with 10 μM inhibitors 4 hours after transfection, as indicated. The cotransfected plasmids used express dominant-negative JNK1 and NF-κB super repressor; the inhibitors used are SB203580 for p38 and PD98059 for ERK. (**D**) Jurkat cells were cotransfected with IFNγluc together with plasmids that express constitutively active MKK6 (MKK6glu), constitutively active IKKβ (IKKCA), MKK7, or JNK1.

IL-12, antibodies to IL-4 (anti–IL-4), and antigen-presenting cells (APCs) and secondary stimulation with ConA. Stimulation of Rac2 knockout T cells under these conditions, however, led to a ~twofold reduction of IFN-γ production (Fig. 4C). Further, when CD4 T cells were stimulated with plate-bound anti-CD3 plus anti-CD28 in the presence of IL-12 and anti-IL-4 for 4 days and restimulated with anti-CD3, IFN-γ production in Rac2 knockout mice was reduced by about two- to threefold compared with that in wild-type mice (Fig. 4D). Thus, the induction of Rac2 expression in T_H1 cells provides these cells with the machinery to generate high-level IFN-γ, and in its absence the levels are reduced.

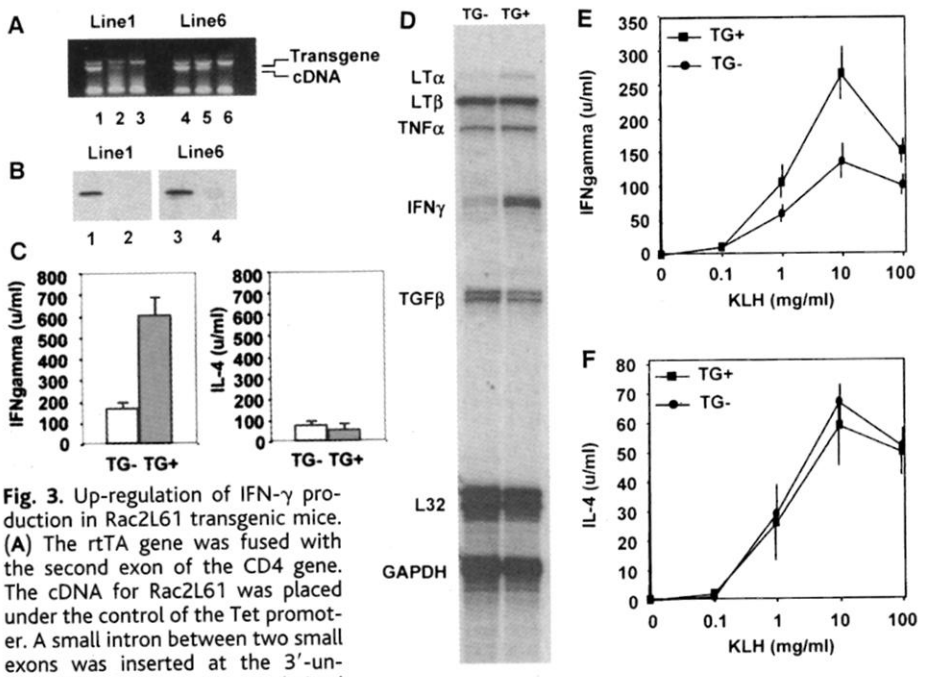

Fig. 3. Up-regulation of IFN-γ production in Rac2L61 transgenic mice. (**A**) The rtTA gene was fused with the second exon of the CD4 gene. The cDNA for Rac2L61 was placed under the control of the Tet promoter. A small intron between two small exons was inserted at the 3′-untranslated region. Primers derived from the second exon and the Rac2L61 coding region are used for screening the transgene mRNA in RT-PCR. T cells were isolated and cultured with APCs, Con A (2.5 μg/ml), IL-2 (30 U/ml), and doxycycline (1 μg/ml) for 2 days. RT-PCR was done with the primers described. Lanes 1 and 4, transgene positive; lanes 2 and lane 5, transgene positive without doxycycline; lanes 3 and 6, controls. The small intron in the 3′-untranslated region of the Rac2L61 gene results in a shorter PCR product from the cDNA than from the transgene. (**B**) T cells from transgene-positive (lanes 1 and 3) and transgene-negative mice (lanes 2 and 4) were treated as described in (A) and analyzed by Western blot with a monoclonal antibody against Myc tag. (**C**) Purified naïve T cells were stimulated in vitro with APCs, ConA, and IL-2 in the presence of doxycycline for 6 days, followed by ribonuclease protection assay. (**D**) Supernatant from day 6 culture described above was used to measure IFN-γ and IL-4 expression by ELISA. (**E** and **F**) Mice were fed with doxycycline food (2 g per kilogram of food) for 7 days and immunized with 50 μg of KLH in CFA in each hind footpad. CD4 T cells in draining lymph nodes were isolated after 9 days and were cultured with KLH for 4 days. The average value of two ELISA readings is shown.

The molecular mechanisms controlling T_H1 cell activation are poorly understood. Our recent reports have demonstrated the involvement of p38 and JNK2 in T_H1 differentiation and IFN-γ production through specific activation of these pathways in T_H1 cells (*7*, *8*, *27*). How this specificity is achieved was previously not clear. Here we show that Rac2 is a T_H1-specific gene that activates IFN-γ production both in vitro and in vivo through concomitant activation of both the NF-κB and p38 pathways. The finding that inactivation of Rac in primary T cells, either by a dominant-negative transgene or by gene targeting of Rac2, inhibits IFN-γ production demonstrates that Rac activation is required for IFN-γ production during normal T cell activation. Rac plays a role in regulating actin polymerization (*5*), and there is evidence supporting its role in T cell activation (*28*); here we demonstrate that Rac directly controls production of a key cytokine involved in T_H cell differentiation. That both the inhibition of Rac signaling through dominant-negative transgenes, and the complete elimination of Rac2 by gene targeting, reduce but do not eliminate production of IFN-γ shows that Rac2 (this report) and the p38 mitogen-activated protein (MAP) [(*7*) and this report] kinase pathways play an amplifying role in the production of high-level IFN-γ at the effector phase of the T cell response. Such amplifying mechanisms are critical for the production of a vigorous immune response in mammals infected by pathogens. This finding and previous results showing the involvement

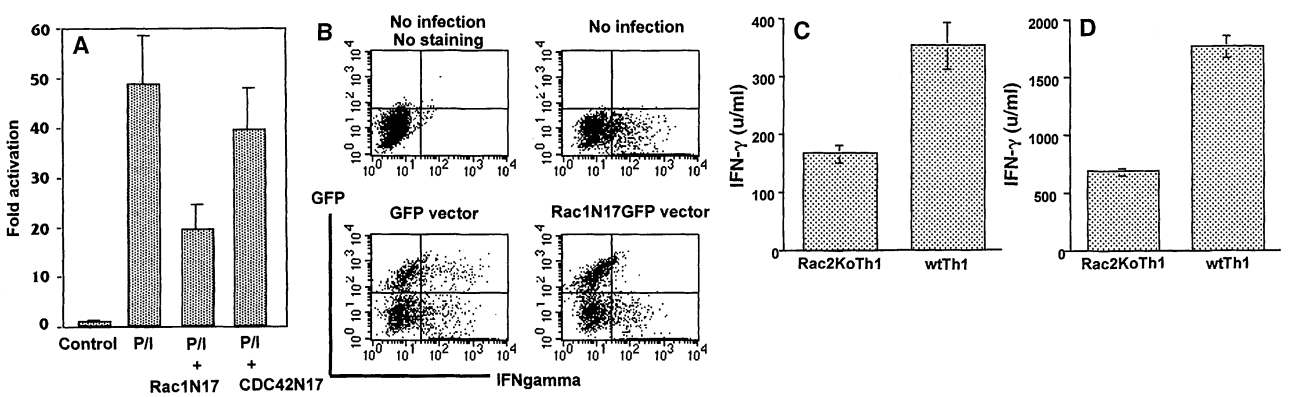

Fig. 4. Dominant-negative Rac or Rac2 deficiency inhibits IFN-γ production in T cells. (**A**) Dual luciferase assay in Jurkat cells was done as described in Fig. 1C. Six micrograms of expression vectors for Rac1N17 or CDC42N17, and 3 μg of IFNγluc were used. (**B**) CD4 T cells from B6 mice stimulated with anti-CD3 and anti-CD28 for 24 hours were infected with recombinant virus supernatant and 5 days later restimulated with PMA-ionomycin, and IFNγ expression was analyzed with intracellular cytokine staining. The internal ribosome entry site (IRES-GFP) element in the vector allows for tracking of infected cells by fluorescence-activated cell sorting. (**C** and **D**) Naïve CD4 T cells from Rac2Ko or wild-type littermate mice were stimulated with ConA plus APC (C) or plate-coated anti-CD3 plus anti-CD28 (D) in the presence of IL-12 and anti-IL-4 for 4 days. Differentiated cells were extensively washed and restimulated with ConA (C) or anti-CD3 (D) for 24 hours. Culture supernatant was collected and assayed for IFN-γ by ELISA.

of Rac in the reorganization of cytoskeletal structure at the site of T cell and APC contact (*29*) demonstrate a dual role for Rac in T cell activation.

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23. Total RNA was isolated with Trizol reagent (Gibco-BRL). The mCK-1 multiprobe template sets (PharMingen) was used for RNase protection assay.
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8 February 2000; accepted 27 April 2000

A Primitive T Cell–Independent Mechanism of Intestinal Mucosal IgA Responses to Commensal Bacteria

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The immunoglobulin A (IgA) is produced to defend mucosal surfaces from environmental organisms, but host defenses against the very heavy load of intestinal commensal microorganisms are poorly understood. The IgA against intestinal commensal bacterial antigens was analyzed; it was not simply "natural antibody" but was specifically induced and responded to antigenic changes within an established gut flora. In contrast to IgA responses against exotoxins, a significant proportion of this specific anti-commensal IgA induction was through a pathway that was independent of T cell help and of follicular lymphoid tissue organization, which may reflect an evolutionarily primitive form of specific immune defense.

The most abundantly produced immunoglobulin in mammals is IgA, which is secreted mainly across mucous membranes. Mucosal immunization of the intestine with adjuvants (*1*) is highly dependent on costimulation (*2*) and T lymphocyte help (*3–5*) within the organized germinal centers of mucosal lymphoid tissues, such as Peyer's patches. Yet paradoxically, despite ineffective mucosal immunization in mouse strains that are T cell–deficient and in those that lack costimulation, intestinal IgA is present (*2*, *6*, *7*), and specific anti-rotaviral responses can still be induced (*8*). We show here that, in contrast to conventional views, a large proportion of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is specifically induced in response to their presence within the microflora, but independently of T cells or germinal center formation. This T cell–independent IgA is derived from B (mostly B1) lymphocytes that develop in the peritoneal compartment (*9–11*) and is distributed diffusely in the intestinal lamina propria. Thus, there exists an important pathway of specific intestinal mucosal IgA induction against antigens of the commensal intestinal microflora, distinct from conventional T cell–dependent IgA found in serum and secretions.

When analyzing normal C57BL/6 mice, maintained under specific pathogen-free (SPF) conditions, we found that they exhibited no specific serum IgG or IgA on Western blots against cell wall proteins of *Enterobacter cloacae* (*12*), the predominant aerobe contained in our SPF intestinal bacterial flora [10^8 colony-forming units (CFU)/gram feces; *Escherichia coli* 10^6 to 10^7 CFU/g feces]. However, they did have specific secretory IgA in the intestinal washings [Fig. 1, A and B; similar results were found with the *E. cloacae* (ATCC 29941 reference strain) or wild-type *E. coli*]. Induction of specific anti-commensal IgA is dependent on the presence of the intestinal microflora (*13*), as there was no binding with equivalent concentrations of intestinal IgA from germ-free C57BL/6 animals (Fig. 1A). When *E. cloacae* preparations were digested with proteinase K (5 μg/ml, 37°C, 30 min) before electrophoresis or after transfer to nitrocellulose, no antibody binding was seen, indicating that protein target antigens were being detected.

Although C57BL/6 SPF mice had no IgG or IgA antibodies specific for commensal bacteria in serum, normal mice could induce specific IgG but not IgA 14 days after intravenous injection of 10^6 CFU of *E. cloacae* (Fig. 1B). No specific IgG was induced after intravenously introduced infection in T cell–deficient mice [T cell receptor (TCR) $\beta^{-/-}\delta^{-/-}$; (*6*), Fig. 1B]. In contrast, when adult gnotobiotic C57BL/6 mice were recolonized with an SPF commensal intestinal flora, which is known to result in temporary bacte-

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