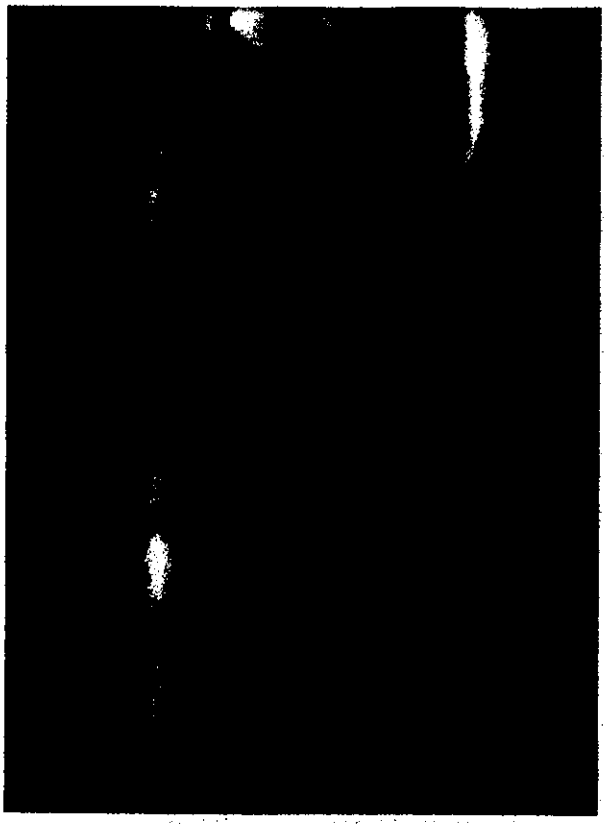


Figure 3. Dual-labeling immunofluorescence staining of synovial tissues with anti–interleukin-22 (green) (**A** and **E**), anti-CD68 (red) (**B**), and anti-CD3 (red) (**F**), and staining of nuclei with Dapi (blue) (**C** and **G**). Merged images are shown in **D** (merger of **A**, **B**, and **C**) and **H** (merger of **E**, **F**, and **G**).

(Figure 5F). In contrast, we did not observe cells double-positive for IL-22R1 and CD68 (Figures 5G–J). IL-22R1 was also expressed in cells in the sublining layer, presumably in the fibroblasts (Figure 5B). Collectively, these results show that IL-22R1 was expressed by synovial fibroblasts and suggest that such expression is constitutive, regardless of inflammatory or noninflammatory conditions.

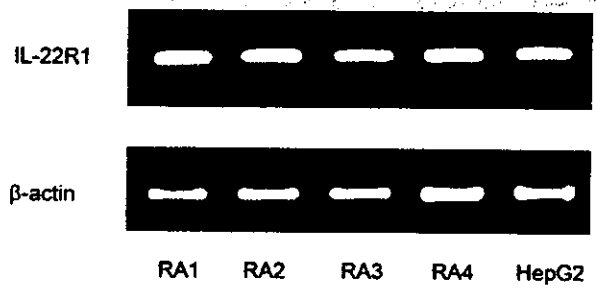


Figure 4. Interleukin-22 receptor 1 (IL-22R1) mRNA expression in rheumatoid arthritis (RA) synovial tissues. Reverse transcription–polymerase chain reaction for IL-22R1 or β-actin was performed using mRNA extracted from the synovial tissues of 4 RA patients (RA1–RA4). HepG2 cells were used as a positive control.

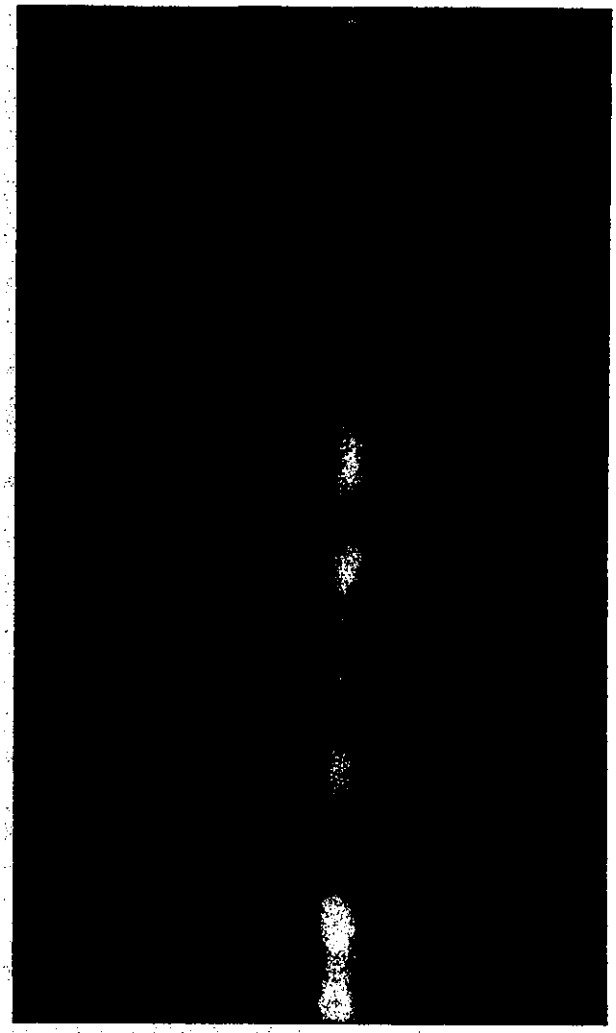


Figure 5. Immunohistologic localization of IL-22R1 in synovial tissue from either patients with RA (**A**, **C–J**) or patients with osteoarthritis (**B**). Dual-labeling immunofluorescence staining of RA synovial tissues (**C–J**) was done with anti–IL-22R1 (green) (**C** and **G**), antivimentin (red) (**D**), and anti-CD68 (red) (**H**). Staining of nuclei was done with Dapi (blue) (**E** and **I**). Merged images are shown in **F** (merger of **C**, **D**, and **E**) and **J** (merger of **G**, **H**, and **I**). See Figure 4 for definitions. (Counterstained with hematoxylin; original magnification × 200.)

IL-22 and IL-22R1 expression in RASF. Immunohistochemical analysis showed that synovial fibroblasts express both IL-22 and IL-22R1. We next exam-

rich3/zar-anr/zar-anr/zar00305/zar5792d05a | meadel | S=5 | 1/10/05 | 15:32 | Art: 04-0348

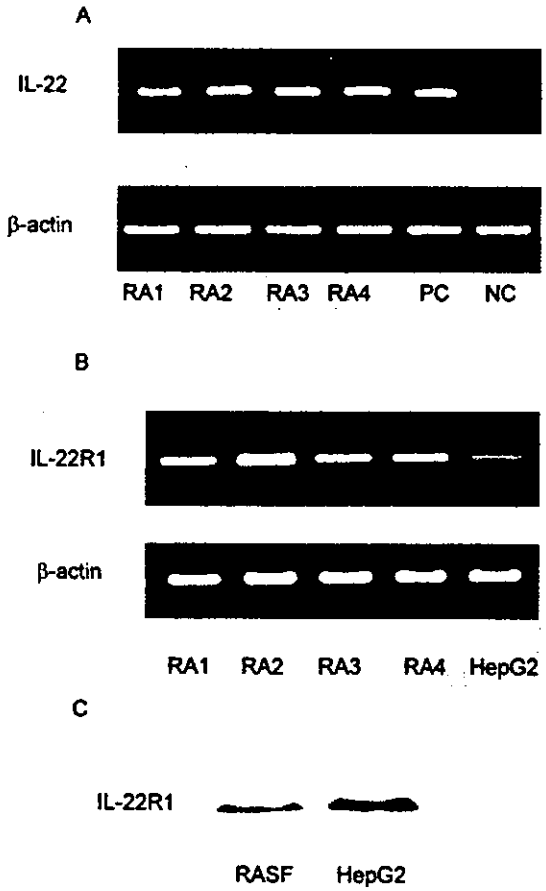


Figure 6. Expression of IL-22 (A) and IL-22R1 (B and C) in synovial fibroblasts established from RA tissue (RASF) obtained from 4 RA patients (RA1–RA4). Reverse transcription–polymerase chain reaction for IL-22 (A), IL-22R1 (B), or β-actin was performed using mRNA extracted from RASF. Western blot was performed using specific antibodies against IL-22R1 (C). Peripheral blood mononuclear cells from normal donors, stimulated with phytohemagglutinin (PC) or without phytohemagglutinin (NC), were used as positive and negative controls, respectively (A). HepG2 was used as a positive control for IL-22R1 (B and C). See Figure 4 for other definitions.

ined whether cultured RASF express IL-22 and IL-22R1. From 4 cell lines of RASF, each established from a different patient's synovial samples, mRNA was isolated and RT-PCR was performed using specific primers against IL-22 or IL-22R1. As shown in Figures 6A and B, mRNA of both IL-22 and IL-22R1 was detected in all 4 RASF samples. PBMCs stimulated either with or without PHA-L were used as positive and negative controls for IL-22, respectively. HepG2 was used as a positive control for IL-22R1. To confirm IL-22R1 expression at the protein level, Western blotting was performed. As shown in Figure 6C, significant levels of IL-22R1 were found to be expressed in RASF, although at lower levels than in HepG2.

Effects of rIL-22 on RASF via synovial fibroblast proliferation and production of chemokines. After confirming that IL-22R1 is expressed in RASF, we examined the effect of rIL-22 on RASF. RASF were incubated with variable concentrations of IL-22 for 72 hours. As shown in Figure 7A, treatment with rIL-22 increased proliferation of RASF in a dose-dependent manner. In contrast, IL-10 or IL-20, another novel IL-10 family cytokine (19) used as a control, did not have an effect on RASF.

MCP-1 is thought to play a pivotal role in macrophage infiltration. Previous studies have shown that MCP-1 could be secreted by RASF and contribute to joint destruction in RA (20). RASF were stimulated with rIL-22 for various lengths of time and MCP-1 production in the supernatants was examined by ELISA. As shown in Figure 7B, IL-22 induced a dose-dependent up-regulation of MCP-1. IL-22 at 100 ng/ml induced a 3-fold increase in MCP-1 above the value in medium controls. MCP-1 mRNA was also examined by real-time PCR. IL-22 induced an up-regulation of MCP-1. A maximal increase in MCP-1 was observed at 12 hours after stimulation (Figure 7C).

ERK-1/2 and p38 MAPK activation by rIL-22. A recent study has shown that IL-22 can activate kinases such as ERK-1/2 and p38 MAPK, activation of which are key events leading to proliferation and MCP-1 production. Therefore, we next examined whether rIL-22 directly induces activation of ERK-1/2 or p38 MAPK. To this end, we performed Western blot using specific antibodies against phosphorylated ERK-1/2 or phosphorylated p38 MAPK. As shown in Figure 8, phospho-ERK-1/2 or phospho–p38 MAPK expression was increased after 30 minutes of stimulation with 100 ng/ml of IL-22.

DISCUSSION

IL-22 is a recently identified IL-10 family cytokine, but its pathophysiologic function is not well known, and neither its expression nor its role in human inflammatory diseases has been explored. In this study, we found that IL-22 was expressed by the synovial fibroblasts and macrophages of the rheumatoid synovium, and that IL-22R1 was expressed by rheumatoid synovial fibroblasts. In vitro, cultured RASF expressed both IL-22 and IL-22R1. Recombinant IL-22 induced prolif-

rich3/zar-anr/zar-anr/zar00305/zar5792d05a | meadel | S=5 | 1/10/05 | 15:32 | Art: 04-0348

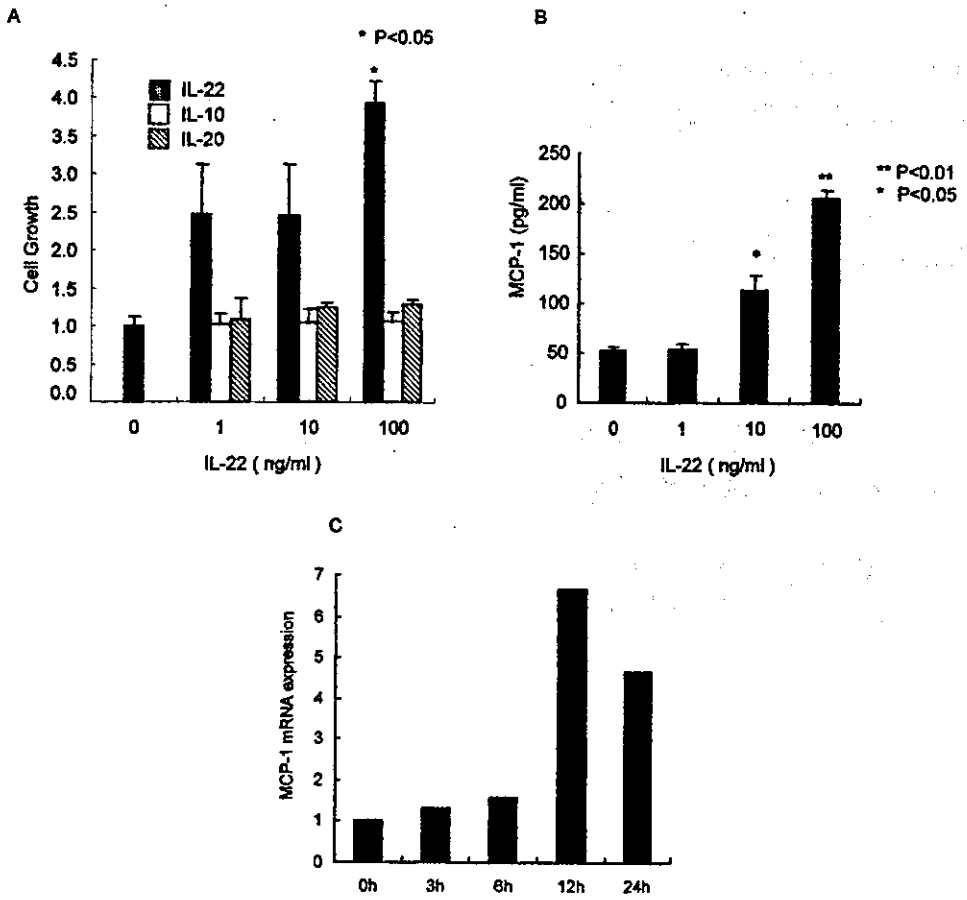


Figure 7. Increased proliferation of RASF and increased RASF production of monocyte chemoattractant protein 1 (MCP-1) by IL-22. RASF were incubated in various concentrations of IL-22, IL-10, or IL-20 for 72 hours. Cell growth was determined by alamer blue assay (**A**). MCP-1 synthesis in the supernatant was determined by enzyme-linked immunosorbent assay after 72 hours (**B**). For mRNA expression, cells were cultured in 100 ng/ml of IL-22 for the indicated time periods. Real-time polymerase chain reaction was performed to determine MCP-1 mRNA expression (**C**). The fold increase in MCP-1 expression at each time point, relative to 0 hours, is shown after correction for β-actin expression. Bars show the mean and SEM results representative of 3 separate experiments. See Figures 4 and 6 for other definitions.

eration and expression of MCP-1 by RASF. Based on these findings, we propose that IL-22, produced by synovial fibroblasts and macrophages, may promote inflammatory responses in RA synovial tissues by inducing synovial fibroblast proliferation and production of chemokines. RASF expressed both IL-22 and IL-22R1, suggesting that IL-22 works in an autocrine as well as a paracrine manner.

IL-22 was originally identified as a cytokine that is produced by T cells stimulated with IL-9 (6). Therefore, we first expected that activated T cells in the rheumatoid synovium might produce IL-22; however, macrophages and synovial fibroblasts, but not T cells, expressed high levels of IL-22. Immunofluorescence studies showed that both vimentin-positive synovial fibroblasts and CD68-positive macrophages were positive for IL-22. Cultured RASF also expressed IL-22, as confirmed by RT-PCR. Previous data have suggested that IL-22 is produced by hematopoetic cells, which in turn affect nonhematopoetic mesenchymal cells (9). In this context, our findings are unique in that IL-22 could be produced not only by hematopoetic cells, but also by mesenchymal cells.

In contrast to IL-22, IL-22R1 expression in the

rich3/zar-anr/zar-anr/zar00305/zar5792d05a | meadel | S=5 | 1/10/05 | 15:32 | Art: 04-0348

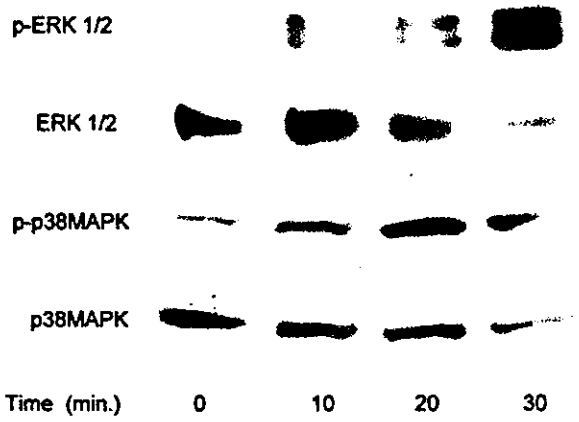


Figure 8. IL-22 induction of the expression of activated ERK-1/2 (p-ERK 1/2) and activated p38 MAPK (p-p38MAPK). RASF were incubated in 100 ng/ml IL-22 for the indicated times, and Western blotting was performed for the expression of p-ERK 1/2, total ERK 1/2, p-p38MAPK, and total p38MAPK. Results are representative of 3 separate experiments. See Figures 4 and 6 for other definitions.

RA synovium, detected by immunohistochemical analysis, was limited to vimentin-positive synovial fibroblasts. Macrophage-lineage cells did not express IL-22R1. In vitro, cultured RASF expressed IL-22R1 both at the mRNA and the protein level. The functional IL-22 receptor complex consists of IL-22R1 and IL-10R2 (10). Since previous studies have shown that RASF express IL-10R2, it is conceivable that RASF could respond to IL-22 in the presence of IL-22R1 (21). Indeed, we found that rIL-22 induced RASF proliferation and production of MCP-1 by RASF. Because synovial fibroblast proliferation and macrophage infiltration by chemokines are key events in the development of synovitis in RA, these are important findings in that they presume the promotion and progression of rheumatoid inflammation by IL-22. Induction of cell growth by IL-22 is shown in other cell types. For example, IL-22 induces proliferation of IL-22R1–transfected Baf3 cells (10). Aggarwal et al showed that IL-22R1 is expressed by the pancreas, and that IL-22 stimulates isolated primary pancreatic acinar cells and the acinar cell line 266-6 to up-regulate mRNA of osteopontin, a chemotactic factor for macrophages (22). Collectively, these previous findings support our hypothesis that IL-22 can induce cell growth and production of molecules with chemotactic activity.

IL-22 has 25% amino acid homology to IL-10 (8). Whereas IL-10 is regarded as an antiinflammatory and immunosuppressive cytokine, previous studies, as well as our own investigations, have suggested that IL-22 works as a proinflammatory cytokine. Molecular mechanisms explaining the difference of the effect between IL-10 and IL-22 have been partially elucidated. IL-10 exerts its function by acting on IL-10 receptors, which consist of IL-10R1 and IL-10R2 (23). Upon IL-10 binding, IL-10R–associated tyrosine kinases (JAK1 and Tyk2) are activated, followed by phosphorylation of STAT-1 and STAT-3 (23). Phosphorylated STAT-1 and STAT-3 translocate regulatory molecules such as SOCS, which results in the antiinflammatory properties of IL-10. Although IL-22 exerts its effect mainly through activation of STAT-1, STAT-3, and STAT-5, a recent study has shown that IL-22 can also activate other important kinases such as ERK-1/2 and p38 MAPK (16). Activation of ERK-1/2 and p38 MAPK are key events leading to proliferation or inflammatory responses, including chemokine production. It is plausible that in our system, IL-22 induced proliferation and MCP-1 production through the activation of ERK and p38 MAPK, respectively. Consistent with this hypothesis, we found that IL-22 induced activation of both ERK-1/2 and p38 MAPK (Figure 8).

Given that IL-22 is a potent inflammatory cytokine in RA, an important question is whether IL-22 can be a therapeutic target. In RA, a variety of cytokines, including TNFα, IL-1β, and IL-6, are thought to contribute to tissue injury. This has been proven by the fact that blocking these cytokines is beneficial for patients with RA (24). The relative contribution of these inflammatory cytokines in RA can differ. In this context, it should be examined whether blocking IL-22 impedes the progression of arthritis in animal models of RA. The naturally occurring soluble molecule IL-22RA, which has blocking activity, has been reported (12,25) and would be an interesting tool for therapeutic application.

In conclusion, our results suggest that IL-22, produced by synovial fibroblasts and macrophages, promotes inflammatory responses in RA by inducing the proliferation of synovial fibroblasts and production of chemokines by synovial fibroblasts. Further studies are necessary to establish the pathophysiologic role of IL-22 in RA.

ACKNOWLEDGMENTS

We thank Ms Rumiko Koitabashi for excellent technical assistance and Dimitrios Boumpas (Divison of Internal Medicine and Rheumatology, University of Crete Medical School) for checking the manuscript.

REFERENCES

1. Firestein GS. Etiology and pathogenesis of rheumatoid arthritis. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, editors. Textbook of rheumatology. 5th ed. Philadelphia: WB Saunders; 1997. p. 851–97.
2. Choy EH, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 2001;344:907–16.
3. Gitter BD, Labus JM, Lees SL, Scheetz ME. Characteristics of human synovial fibroblast activation by IL-1 β and TNFα. Immunology 1989;66:196–200.
4. Furst DE, Schiff MH, Fleischmann RM, Strand V, Birbara CA, Compagnone D, et al. Adalimumab, a fully human anti tumor necrosis factor-α monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis). J Rheumatol 2003;30:2563–71.
5. Tanaka Y, Otsuka T, Hotokebuchi T, Miyahara H, Nakashima H, Kuga S, et al. Effect of IL-10 on collagen-induced arthritis in mice. Inflamm Res 1996;45:283–8.
6. Katsikis PD, Chu CQ, Brennan FM, Maini RN, Feldmann M. Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 1994;179:1517–27.
7. Dumoutier L, Louahed J, Renauld JC. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. J Immunol 2000;164:1814–9.
8. Dumoutier L, van Roost E, Colau D, Renauld JC. Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. Proc Natl Acad Sci U S A 2000;97:10144–9.
9. Wolk K, Kunz S, Asadullah K, Sabat R. Cutting edge: immune cells as sources and targets of the IL-10 family members? J Immunol 2002;168:5397–402.
10. Kotenko SV, Izotova LS, Mirochnitchenko OV, Esterova E, Dickensheets H, Donnelly RP, et al. Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rβ) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. J Biol Chem 2001;276:2725–32.
11. Xie MH, Aggarwal S, Ho WH, Foster J, Zhang Z, Stinson J, et al. Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. J Biol Chem 2000;275:31335–9.
12. Kotenko SV, Izotova LS, Mirochnitchenko OV, Esterova E, Dickensheets H, Donnelly RP, et al. Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity. J Immunol 2001;166:7096–103.
13. Dumoutier L, Lejeune D, Colau D, Renauld JC. Cloning and characterization of IL-22 binding protein, a natural antagonist of IL-10-related T cell-derived inducible factor/IL-22. J Immunol 2001;166:7090–5.
14. Tachiiri A, Imamura R, Wang Y, Fukui M, Umemura M, Suda T. Genomic structure and inducible expression of the IL-22 receptor α chain in mice. Genes Immun 2003;4:153–9.
15. Lecart S, Morel F, Noraz N, Pene J, Garcia M, Boniface K, et al. IL-22, in contrast to IL-10, does not induce Ig production, due to absence of a functional IL-22 receptor on activated human B cells. Int Immunol 2002;14:1351–6.
16. Lejeune D, Dumoutier L, Constantinescu S, Kruijer W, Schuringa JJ, Renauld JC. Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line: pathways that are shared with and distinct from IL-10. J Biol Chem 2002;277:33676–82.
17. Ota F, Maeshima A, Yamashita S, Ikeuchi H, Kaneko Y, Kuroiwa T, et al. Activin A induces cell proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis Rheum 2003;48: 2442–9.
18. Tanaka T, Kuroiwa T, Ikeuchi H, Ota F, Kaneko Y, Ueki K, et al. Human platelets stimulate mesangial cells to produce monocyte chemoattractant protein-1 via the CD40/CD40 ligand pathway and may amplify glomerular injury. J Am Soc Nephrol 2002;13: 2488–96.
19. Blumberg H, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S, et al. Interleukin 20: discovery, receptor identification, and role in epidermal function. Cell 2001;104:9–19.
20. Koch AE, Kunkel SL, Harlow LA, Johnson B, Evanoff HL, Haines GK, et al. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. J Clin Invest 1992;90:772–9.
21. Ritchlin C, Haas-Smith SA. Expression of interleukin 10 mRNA and protein by synovial fibroblastoid cells. J Rheumatol 2001;28: 698–705.
22. Aggarwal S, Xie MH, Maruoka M, Foster J, Gurney AL. Acinar cells of the pancreas are a target of interleukin-22. J Interferon Cytokine Res 2001;21:1047–53.
23. Kotenko SV, Krause CD, Izotova LS, Pollack BP, Wu W, Pestka S. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. EMBO J 1997;16: 5894–903.
24. Giraldo P, Regales L, Lavado A, Tovar V, Garcia-Diaz A, Jimenez E, et al. IL-22 the mouse tyrosinase gene: structural and functional studies in transgenic mice. Pigment Cell Res 2003;16:582. AQ: 2
25. Xu W, Presnell SR, Parrish-Novak J, Kindsvogel W, Jaspers S, Chen Z, et al. A soluble class II cytokine receptor, IL-22RA2, is a naturally occurring IL-22 antagonist. Proc Natl Acad Sci U S A 2001;98:9511–6.

特集　リウマチ性疾患とシグナル伝達分子異常

SLE患者T細胞における CD 28分子の発現とシグナル伝達異常

針谷正祥*　川本　学**

T細胞の完全な活性化には，T細胞レセプターおよび副刺激分子の両者を介したシグナルが必要とされている．CD 28は代表的な副刺激分子であり，そのシグナル伝達機構として，フォスファチジルイノシチジル-3キナーゼ，Vav，TECが重要である．全身性エリテマトーデス（SLE）モデルマウスでは，CD 28-CD 80/CD 86の阻害による治療効果が，ヒトSLE患者ではCD 28およびCD 80/CD 86の発現異常・機能異常が報告されており，CD 28のSLE病態形成における重要性が示唆されている．

はじめに

全身性エリテマトーデス（systemic lupus erythematosus：SLE）では自己反応性T細胞の異常がその病態に関与すると考えられており，各種の細胞表面分子および細胞内分子の発現・機能異常が報告されている．一般にT細胞の完全な活性化には，T細胞レセプター（T cell receptor：TCR）が抗原提示細胞上に提示されたペプチドと主要組織適合性抗原（major histocompatibility complex：MHC）の複合体を認識することによりもたらされる抗原特異的シグナル（シグナル1）と，副刺激分子によりもたらされる抗原非特異的シグナル（シグナル2）の両者が必要とされる．シグナル2の非存在下でT細胞が抗原提示を受けるとT細胞は不応答状態（anergy）に陥る．また，シグナル2には刺激性シグナルと抑制性シグナルの2種類が存在し，そのバランスがT細胞の運命を決定するうえで重要と考えられる[1]．

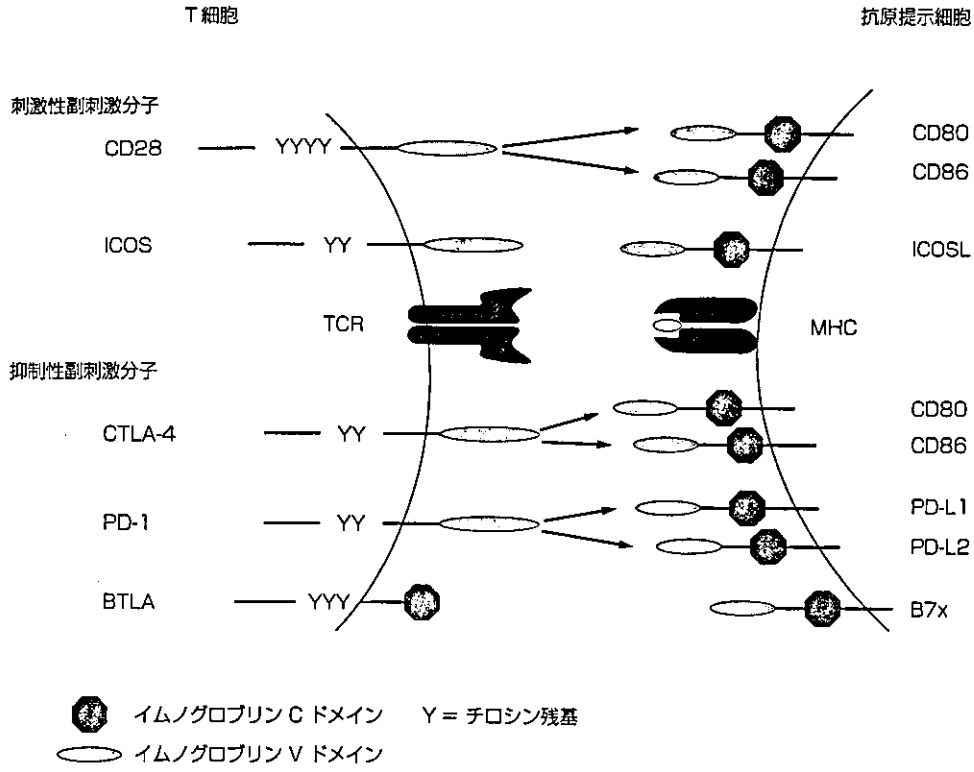
CD 28分子は代表的な副刺激分子であり，SLEモデルマウスおよびSLE患者末梢血リンパ球における解析結果が報告されている．本稿では，CD 28分子の構造・機能・SLEの病態形成における役割について諸家の報告を概説する．

KEY WORDS
CD 28
B 7
副刺激分子
全身性エリテマトーデス

*HARIGAI Masayoshi/東京医科歯科大学医学部臨床試験管理センター/膠原病・リウマチ内科
**KAWAMOTO Manabu/東京女子医科大学附属膠原病リウマチ痛風センター

1 CD 28分子の構造

CD 28ファミリーには現在のところ，CD 28，CTLA-4（cytotoxic T lymphocyte associated-4），ICOS（inducible costimulator），PD-1（programmed cell death-1），BTLA（B and T lymphocyte attenuator）が属している．各分子のレセプターとしてCD 28およびCTLA-4にはCD 80およびCD 86が，ICOSにはICOSリガンド（ICOSL）が，PD-1にはPD-L 1およびPD-L 2が，BTLAにはB 7 xが同定されている[1)2)]（図❶）．CD 28は細胞外に1個のイムノグロブリンVドメインを有す



図❶ CD 28 ファミリー分子および B 7 ファミリー分子
刺激性副刺激分子，抑制性副刺激分子に分類して示した．CD 28 ファミリー分子が 2 つ以上の B 7 ファミリー分子と結合する場合は矢印で示してある．図には示していないが，結合するパートナーが同定されていない B 7 ファミリー分子として B 7-H 3 が報告されている．また，PD-L 1 および PD-L 2 に共通する第 2 のパートナー(CD 28 ファミリー分子)が存在する可能性が考えられている．

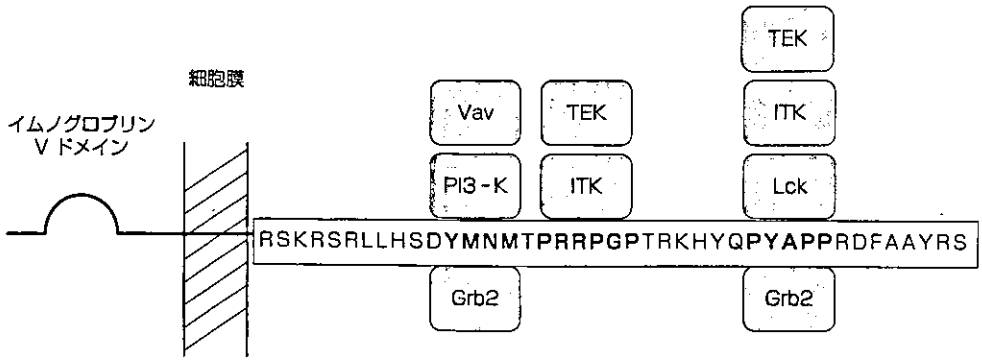
る分子量 44 kDa の I 型膜蛋白で，2 分子が会合して二量体を形成している．CD 28 の細胞外ドメインには Met-Tyr-Phe-Phe-Tyr (MYPPY) というアミノ酸配列があり，CD 80 および CD 86 はこのアミノ酸配列を認識して CD 28 に結合する．CD 28 の細胞質ドメインには，Tyr-Met-Asp-Met (YMNM) モチーフが存在し，この部分にフォスファチジルイノシタイド-3 キナーゼ (phosphatidylinositide 3-kinase：PI 3-K) が結合することが知られている．さらに，図❷に示すようなシグナル伝達分子が CD 28 の細胞質ドメインに結合し，T 細胞活性化に関連している[3)4)]．

❷ CD 28 分子のシグナル伝達機構

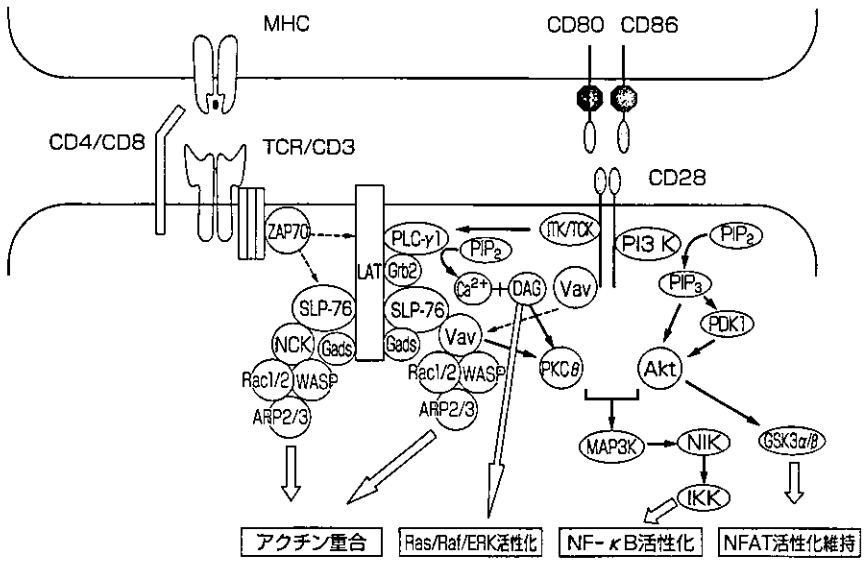
CD 28 を介するシグナルは，T 細胞活性化の閾値を低下させ，活性化に必要な APC 上の MHC-ペプチド複合体の数を減少させ，最終的には抗原刺激に対する T 細胞の反応性を増強・維持するはたらきを有する．その分子機序として，CD 28 からのシグナルによりインターロイキン (IL)-2 遺伝子のクロマチンリモデリングと脱メチル化が誘導され，T 細胞周期の進展，転写因子(NFAT，NF-κB，AP 1，Myc) 活性化，BCL-XL の発現誘導，サイトカイン・ケモカイン(IL-2，IL-4，IFN-γ，CCL 3 など) 遺伝子の発現誘導，他の副刺激分子 (CD 154，OX 40，ICOS，CTLA-4 など)の発現誘導などが知られている．CD 28 のシグナル伝達機構として以下の 3 つの経路が特に重要である[3)~5)](図❸)．

1．PI 3-K 活性化

CD 28 の架橋によりリン酸化された YMNM モチーフに PI 3-K が p 85 サブユニットを介して結合し，活性化を受ける．PI 3-K はフォスファチジルイノシトール-4,5-ビスフォスフェートからフォスファチジルイノシ



図❷ CD 28 の細胞質ドメインのアミノ酸配列と結合するシグナル伝達関連因子(Acuto O *et al*, 2003[3], Rudd CE *et al*, 2003[4]より改変引用)
ヒト CD 28 分子の細胞質ドメインのアミノ酸配列を示す．太字のアミノ酸は，各分子の結合に重要な配列を示す．PI 3-K は YMNM モチーフに結合し活性化される．活性化 PI 3-K が Vav をリン酸化する．ITK および TEK は 2ヵ所のプロリンリッチ領域に結合しうるが，おもに最初のプロリンリッチ領域がこれらの結合と活性化に関与する．



図❸ CD 28 副刺激存在下の TCR シグナル伝達経路
CD 28 刺激により，細胞質ドメインに PI 3-K が結合し，PIP 3 産生，Akt 活性化をもたらす．活性化 Akt は GSK 3 α/β の活性化をつうじて NFAT の核外輸送を阻害し，NFAT の活性維持に寄与する．また，CD 28 刺激により活性化された Vav は TCR 刺激により形成された LAT-SLP 76 複合体に結合し，Rac 1/2，WASP，ARP 2/3 を介してアクチン重合を誘導する．CD 28 刺激により ITK，TCK は CD 28 の細胞質ドメインに結合し，活性化される．このほかに ITK，TCK を活性化する経路として，Lck・Fyn によるチロシンリン酸化，PIP_3による活性化が知られている．活性化 ITK および TCK は PLCγ-1 を活性化し，細胞内 Ca^{2+}およびジアシルグリセロール (DAG) 濃度の上昇をもたらす．DAG あるいは Vav により PKCθ が活性化を受け，PI 3-K により活性化された Akt とともに NF-κB 経路を活性化する．また DAG は Ras/Raf/ERK 経路の活性化，PKC の活性化にも関連する．

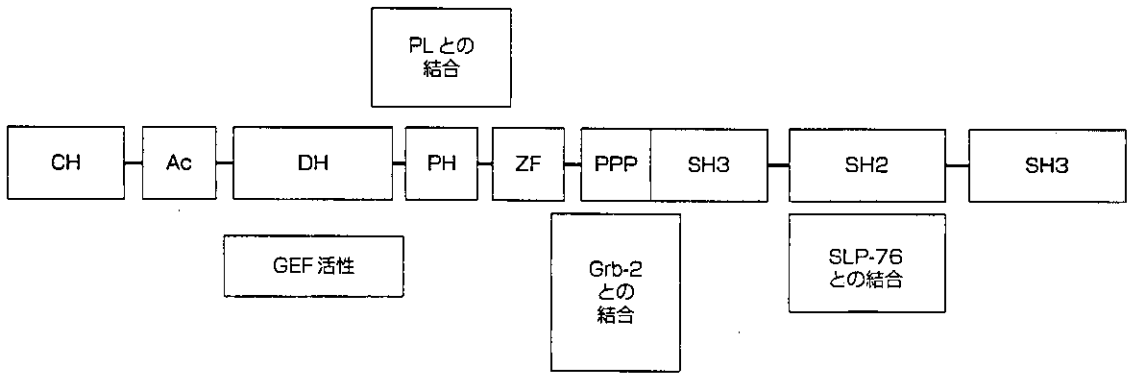
トール-3,4,5-トリフォスフェート (PIP_3) およびフォスファチジルイノシトール-3,4-ビスフォスフェートを産生する．PIP_3 は Akt を活性化し，活性化 Akt はグリコーゲンシンテースキナーゼ 3 α (GSK 3 α) および GSK 3 β を活性化する．Akt は TCR により活性化されたプロテインキナーゼ Cθ (protein kinase Cθ：PKCθ) とともに NF-κB 経路を活性化する．また，活性化 GSK 3 α/GSK 3 β は NFAT の核から細胞質への移行を抑制する．このようにして，PI 3-K は転写因子の活性化を介して，T 細胞反応性の増強・維持に関与する(図❸)．

2．Vav 活性化

CD 28 の架橋により Vav が活性化を受ける．Vav は C 末端に 2 個の SH 3 ドメインと 1 個の SH 2 ドメインを有するアダプター蛋白で，N 末端側には Vav の活性に重要な複数の Tyr 残基を含む酸性ドメインを有する (図❹)．CD 28 の細胞質ドメインに結合する $p56^{lck}$ により Vav の^{174}Tyr がリン酸化を受ける可能性が考えられている．Vav の DBL ホモロジードメインは GEF (guanine nucleotide exchange) 活性を有し，Rac 1，Rac 2 などの Rho ファミリー蛋白の GTPase 活性を刺激する．一方 TCR 刺激により ZAP 70 (Zeta-associated protein 70 kDa) が活性化され，活性化 ZAP 70 により GEM (glycolipid-enriched membrane microdomain) に存在する足場蛋白質である LAT (linker for activated T cells) および SLP 76 (SH 2 domain containing leukocyte protein of 76 kDa) がリン酸化を受け，Gads, Grb 2, フォスフォリパーゼ Cγ-1 (phospholipase Cγ-1：PLCγ-1) などとともに複合体を形成する．この LAT-SLP 76 複合体は TCR シグナル伝達の重要な要素であり，Nck および CD 28 の架橋により活性化を受けた Vav も LAT-SLP 76 複合体に結合し，Rac 1/2, WASP (Wiskott-Aldrich syndrome protein), ARP 2/3 を介してアクチンの重合を誘導すると同時に GEM の会合に関与する(図❸)．

3．TEC キナーゼ活性化

TEC および ITK (IL-2-inducible T-cell kinase) は TCR 刺激により活性化を受けるチロシンキナーゼである．活性化 TEC および ITK は PLCγ-1 を活性化し，細胞内 Ca 濃度上昇，Ras/Raf/ERK 経路活性化，Rac 1/2, WASP, ARP 2/3 を介したアクチン重合を引き起こす(図❸)．図❷に示すように，CD 28 分子の細胞質ドメインには TEC および ITK の結合部位が存在し，CD 28 の架橋により TEC および ITK がこの部位にリクルートされる．しかし TCR 刺激の場合と異なり，CD 28 の架橋のみでは PLCγ-1 活性化，細胞内 Ca 濃度上昇，Ras/Raf/ERK 経路活性化，アクチン重合などは誘導されない．したがって，CD 28 の架橋による TEC および ITK 活性化は，同時に受ける TCR 刺激依存性にその機能が発揮されると考えられている．



図❹ Vav の構造および機能 (Rudd CE *et al*, 2003[4]より改変引用)
T 細胞活性化の重要なアダプター分子である Vav は，カルポニンホモロジードメイン(CH)，酸性ドメイン (Ac)，DBL-ホモロジードメイン (DH)，PH ドメイン，Zn フィンガー様ドメイン (ZF)，プロリンリッチ領域(PPP)，2 個の SH 3 ドメイン，1 個の SH 2 ドメインから構成される．T 細胞活性化における各部位の重要な機能についても示した．PL＝フォスフォリピッド

③ SLEモデルマウスにおけるCD 28-CD 80/86の役割

SLEにおけるCD 28-CD 80/CD 86相互作用の重要性が，SLEモデルマウスを使って検討されている．マウスCTLA-4と免疫グロブリンのFc部分の融合蛋白(mCTLA-4-Ig)を作成し，SLEモデルマウスであるNZB/NZW F 1マウスに投与したところ，自己抗体産生が抑制され生存期間が延長した．mCTLA-4-Igの投与時期をループスが発症した時期まで遅らせても，これらの効果は認められ，CD 28-CD 80/CD 86の相互作用がNZB/NZW F 1マウスの腎病変発症および進展に重要であることが示された[6]．一方，NZB/NZW F 1マウスを抗CD 80抗体，抗CD 86抗体で加療したところ，抗CD 80抗体単独投与では血清中の自己抗体価が抑制されなかったが，抗CD 86抗体単独投与で血清中のIgG_1およびIgG_{2b}抗2本鎖DNA抗体(抗dsDNA抗体)価が低下した．両者の組み合わせによりIgG_{2a}抗dsDNA抗体価も低下し，腎病変の軽減と生存率の向上が認められた[7]．Daikhら[8]はNZB/NZW F 1マウスに対するCTLA-4-Igと抗CD 40抗体の短期間同時投与の効果を検討した．CTLA-4-Igをループス病変の発症時期に短期間投与するだけでは一過性の改善効果しか得られなかったが，CTLA-4-Igと同時に抗CD 40抗体を2週間投与したところ長期的な自己抗体価の低下と生存率の向上が認められた[8]．

他のSLEモデルマウスであるMRL/lprマウスにおいてCD 28遺伝子を欠損させたところ，リンパ節腫脹は軽減したが，脾腫が増大し$B220^+TCR\alpha\beta^+$T細胞が増加した．血清中IgG抗dsDNA抗体・IgGリウマトイド因子は低下し，腎病変の軽減が認められた．また腎血管炎・関節炎も消失した[9]．

以上のように，CD 28-CD 80/CD 86相互作用は自己抗体とくに疾患特異的な抗2本鎖DNA抗体の産生に寄与し，腎病変の成立にかかわっていると考えられる．

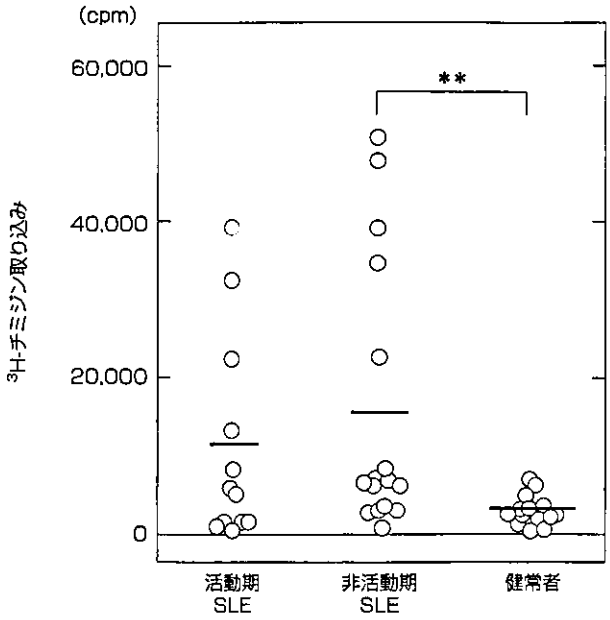
④ SLEにおけるCD28分子，CD 80/CD 86分子の発現

SLE患者末梢血T細胞におけるCD 28発現を解析した結果では，$CD 4^+CD 28^+$T細胞，$CD 8^+CD 28^+$T細胞の割合および絶対数が健常者に比較して低下していたと報告されている[10][11]．一方，SLEにおいて抗dsDNA抗体産生扶助能力を有すると報告されている$CD 3^+CD 4^-CD 8^-$〔DN (double negative)〕$TCR\alpha\beta^+$T細胞の割合はSLE患者で有意に高く，CD 28を含む活性化マーカー(CTLA-4，HLA-DR，CD 69)陽性率が有意に上昇していた[12]．SLEの皮疹部においては，抗原提示細胞がCD 80/CD 86を発現し，皮膚浸潤T細胞がCD 28分子を発現していた．治療により，皮疹部のCD 80/CD 86発現は低下した[13]．細胞表面のCD 28分子はsheddingを受け，可溶性CD 28分子となり循環血液中に存在する．SLE患者・シェーグレン症候群患者血清中では可溶性CD 28分子が健常者よりも高濃度で存在することも報告されている[14]．

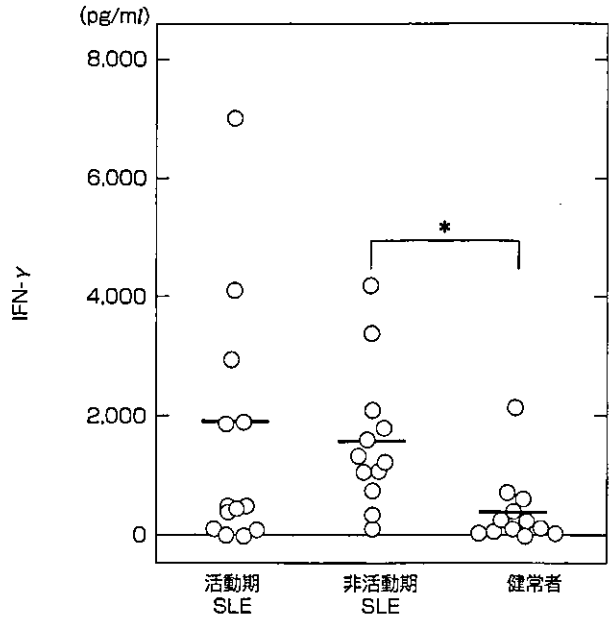
⑤ SLEにおけるCD 28分子の機能異常

SLE患者末梢血T細胞の機能解析をおこなう実験では，TCRを介さずに直接下流のシグナル伝達機構を活性化させる刺激〔PMA (phorbol myristate acetate)＋カルシウムイオノフォアなど〕を用いる場合と，TCRを介した刺激(抗CD 3抗体＋抗CD 28抗体など)を用いる場合がある．SLEではTCR ζ鎖の発現低下を有する症例が認められ，TCR刺激に対する反応性異常が報告されているため，上記の2種類の刺激方法による解析結果は必ずしも一致しない．また，T細胞を精製して用いた場合と抗原提示細胞（単球・B細胞）を含む末梢血リンパ球を用いた場合では，当然のことながら異なった結果が出る可能性がある．上述のようにCD 28分子を介した副刺激存在下にT細胞が活性化されると，T細胞の増殖，転写因子活性化，サイトカイン・ケモカイン産生などの反応が認められる．これらの反応を指標として，SLEにおけるCD 28分子の機能解析結果が報告されている．

Alarcon-Segoviaらのグループ[11]はSLE末梢血T細胞を3日間抗CD 3抗体＋抗CD 28抗体で刺激し，SLEでは有意に高い^{3}H-チミジン取り込みが得られることを報告している．われわれの検討でも，SLE末梢血T細胞は抗CD 3抗体＋抗CD 28抗体刺激に強い増殖反応を示すことが確認されている．SLE末梢血T細胞を無刺激



図❺ SLE 患者末梢血 T 細胞の抗 CD 3 抗体＋抗 CD 28 抗体刺激に対する ^{3}H-チミジン取り込みの亢進
活動期 SLE 患者，非活動期 SLE 患者，健常者の末梢血 T 細胞を抗 CD 3 抗体＋抗 CD 28 抗体にて 64 時間刺激後，8 時間 ^{3}H-チミジンでパルスした．非活動期 SLE では健常者に比較し有意に高い ^{3}H-チミジン取り込みが認められた．＊＊ $p<0.01$



図❻ SLE 患者末梢血 T 細胞の抗 CD 3 抗体＋抗 CD 28 抗体刺激に対する IFN-γ 産生の亢進
活動期 SLE 患者，非活動期 SLE 患者，健常者の末梢血 T 細胞を抗 CD 3 抗体＋抗 CD 28 抗体にて 72 時間刺激後，培養上清中の IFN-γ を ELISA で測定した．非活動期 SLE では健常者に比較し有意に高い IFN-γ 産生が認められた．＊ $p<0.05$

または抗 CD 3 抗体＋抗 CD 28 抗体刺激で 64 時間培養し，8 時間の ^{3}H-チミジン取り込みを測定した結果を図❺に示す．健常者に比較し，非活動期 SLE では ^{3}H-チミジン取り込みが有意に亢進していた．活動期 SLE でも平均値は上昇していたが，抗 CD 3 抗体＋抗 CD 28 抗体刺激に応答性のきわめて低い患者が少数存在したために，統計学的有意差には至らなかった．同時に測定した 72 時間刺激培養上清中の IFN-γ 濃度も非活動期 SLE では健常者よりも有意に高く，活動期 SLE では平均値は上昇していたものの，^{3}H-チミジン取り込みと同様に統計学的有意差には至らなかった[15]（図❻）．Tsokos らのグループ[16]は SLE 末梢血 T 細胞を抗 CD 3 抗体＋抗 CD 28 抗体または PMA＋カルシウムイオノフォアで刺激後の NF-κB 活性が低下しており，とくに p 65 の発現低下が NF-κB 活性化障害の原因となっていると報告している[16]．末梢血 $CD28^+$T 細胞が SLE では減少していると報告したグループは，$CD28^-$T 細胞の抗 CD 3 抗体に対する反応性が $CD28^+$T 細胞の反応性と比較して低下していると報告している[10]．

一方，CD 80/CD 86 の機能異常についても報告されている．SLE 末梢血リンパ球を抗 CD 2 抗体で刺激したところ，抗 CD 3 抗体あるいは PHA で刺激した場合よりも増殖反応が低下しており，その低下は抗 CD 28 抗体の添加によりほぼ消失した．また末梢血リンパ球から付着細胞を除去し，健常者の単球・B 細胞を加えることにより抗 CD 2 抗体刺激に対する増殖反応が改善したことから，SLE では抗原提示細胞と T 細胞間の副刺激経路が障害されていると考えられた[17]．

このように，ヒト SLE における CD 28 分子の機能解析結果は報告者により必ずしも一致しない部分がある．その理由として SLE の多様性による解析対象症例の差異，実験方法の違いなどが考えられる．

おわりに

以上述べてきたように，CD 28-CD 80/CD 86 相互作用は SLE の病態形成に重要なはたらきをしている．モデルマウスではこの相互作用が抗 dsDNA 抗体産生に寄与し，ヒト SLE では T 細胞機能異常にかかわっていると

考えられる．CD 28副刺激がTCR刺激の強さを調節するのか，刺激の質を変えるのか，両者なのかは現在も議論の最中であり，この点はヒトSLE末梢血T細胞の抗CD 3抗体＋抗CD 28抗体刺激に対する応答異常を考察するうえでも大変興味深く，今後の研究の進展が期待される．

文　献

1) Sharpe AH, Freeman GJ：The B 7-CD 28 superfamily. *Nat Rev Immunol* **2**：116-126, 2002
2) Watanabe N, Gavrieli M, Sedy JR *et al*：BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* **4**：670-679, 2003
3) Acuto O, Michel F：CD 28-mediated co-stimulation：a quantitative support for TCR signalling. *Nat Rev Immunol* **3**：939-951, 2003
4) Rudd CE, Raab M：Independent CD 28 signaling via VAV and SLP-76：a model for *in trans* costimulation. *Immunol Rev* **192**：32-41, 2003
5) Kane LP, Weiss A：The PI-3 kinase/Akt pathway and T cell activation：pleiotropic pathways downstream of PIP 3. *Immunol Rev* **192**：7-20, 2003
6) Finck BK, Linsley PS, Wofsy D：Treatment of murine lupus with CTLA 4 Ig. *Science* **265**：1225-1227, 1994
7) Nakajima A, Azuma M, Kodera S *et al*：Preferential dependence of autoantibody production in murine lupus on CD 86 costimulatory molecule. *Eur J Immunol* **25**：3060-3069, 1995
8) Daikh DI, Finck BK, Linsley PS *et al*：Long-term inhibition of murine lupus by brief simultaneous blockade of the B 7/CD 28 and CD 40/gp 39 costimulation pathways. *J Immunol* **159**：3104-3108, 1997
9) Tada Y, Nagasawa K, Ho A *et al*：Role of the costimulatory molecule CD 28 in the development of lupus in MRL/lpr mice. *J Immunol* **163**：3153-3159, 1999
10) Kaneko H, Saito K, Hashimoto H *et al*：Preferential elimination of CD 28＋ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis. *Clin Exp Immunol* **106**：218-229, 1996
11) Alvarado C, Alcocer-Varela J, Llorente L *et al*：Effect of CD 28 antibody on T cells from patients with systemic lupus erythematosus. *J Autoimmun* **7**：763-773, 1994
12) Anand A, Dean GS, Quereshi K *et al*：Characterization of CD 3＋CD 4-CD 8-(double negative) T cells in patients with systemic lupus erythematosus：activation markers. *Lupus* **11**：493-500, 2002
13) Denfeld RW, Kind P, Sontheimer RD *et al*：*In situ* expression of B 7 and CD 28 receptor families in skin lesions of patients with lupus erythematosus. *Arthritis Rheum* **40**：814-821, 1997
14) Hebbar M, Jeannin P, Magistrelli G *et al*：Detection of circulating soluble CD 28 in patients with systemic lupus erythematosus, primary Sjögren's syndrome and systemic sclerosis. *Clin Exp Immunol* **136**：388-392, 2004
15) Kawamoto M, Harigai M, Hara M *et al*：Overexpression of inducible co-stimulator (ICOS) on peripheral blood T cells and its contribution to abnormal T cell function in patients with systemic lupus erythematosus. *Arthritis Rheum* **46** (Supple)：S 282, 2002
16) Wong HK, Kammer GM, Dennis G *et al*：Abnormal NF-kappa B activity in T lymphocytes from patients with systemic lupus erythematosus is associated with decreased p 65-RelA protein expression. *J Immunol* **163**：1682-1689, 1999
17) Horwitz DA, Tang FL, Stimmler MM *et al*：Decreased T cell response to anti-CD 2 in systemic lupus erythematosus and reversal by anti-CD 28：evidence for impaired T cell-accessory cell interaction. *Arthritis Rheum* **40**：822-833, 1997

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 48, Issue of November 26, pp. 50102–50109, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

The Receptor Tyrosine Kinase Ror2 Associates with and Is Activated by Casein Kinase Iε*[S]

Received for publication, August 6, 2004, and in revised form, September 14, 2004
Published, JBC Papers in Press, September 15, 2004, DOI 10.1074/jbc.M409039200

Shuichi Kani‡§¶, Isao Oishi‡§, Hiroyuki Yamamoto‡, Akinori Yoda‡, Hiroaki Suzuki‡, Akira Nomachi‡, Kengo Iozumi‡, Michiru Nishita‡, Akira Kikuchi‖, Toru Takumi, and Yasuhiro Minami‡ ‡‡**

*From the ‡Department of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, Kobe 650-0017, Japan, the ‖Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan, and the **Osaka Bioscience Institute, Osaka 565-0874, Japan*

Ror2, a member of the mammalian Ror family of receptor tyrosine kinases, plays important roles in developmental morphogenesis, although the mechanism underlying activation of Ror2 remains largely elusive. We show that when expressed in mammalian cells, Ror2 associates with casein kinase Iε (CKIε), a crucial regulator of Wnt signaling. This association occurs primarily via the cytoplasmic C-terminal proline-rich domain of Ror2. We also show that Ror2 is phosphorylated by CKIε on serine/threonine residues, in its C-terminal serine/threonine-rich 2 domain, resulting in autophosphorylation of Ror2 on tyrosine residues. Furthermore, it was found that association of Ror2 with CKIε is required for its serine/threonine phosphorylation by CKIε. Site-directed mutagenesis of tyrosine residues in Ror2 reveals that the sites of phosphorylation are contained among the five tyrosine residues in the proline-rich domain but not among the four tyrosine residues in the tyrosine kinase domain. Moreover, we show that in mammalian cells, CKIε-mediated phosphorylation of Ror2 on serine/threonine and tyrosine residues is followed by the tyrosine phosphorylation of G protein-coupled receptor kinase 2, a kinase with a developmental expression pattern that is remarkably similar to that of Ror2. Intriguingly, a mutant of Ror2 lacking five tyrosine residues, including the autophosphorylation sites, fails to tyrosine phosphorylate G protein-coupled receptor kinase 2. This indicates that autophosphorylation of Ror2 is required for full activation of its tyrosine kinase activity. These findings demonstrate a novel role for CKIε in the regulation of Ror2 tyrosine kinase.

Receptor tyrosine kinases (RTKs)[1] play important roles in developmental morphogenesis by regulating growth, differentiation, motility, adhesion, and death of many types of cells (1). It has been well documented that the interactions of RTKs with their cognate ligands trigger their dimerization or oligomerization, resulting in tyrosine autophosphorylation and tyrosine kinase activation of RTKs. This induces various intracellular signaling events. In contrast, it has been reported that tyrosine autophosphorylation and the tyrosine kinase activities of several RTKs, including the insulin and epidermal growth factor receptors, can be negatively regulated by ligand-independent transphosphorylation of these RTKs by cytoplasmic serine/threonine kinases (2–8). However, little is known about the positive regulation of RTK tyrosine autophosphorylation and tyrosine kinase activation caused by cytoplasmic serine/threonine kinases.

The mammalian Ror family of RTKs, consisting of two structurally related proteins, Ror1 and Ror2, are orphan RTKs, characterized by several conserved domain structures, the extracellular Frizzled-like cysteine-rich domains, and the membrane-proximal Kringle domains that are assumed to mediate protein-protein interactions (9–13). It has been reported that in nematodes and mammals, Ror family RTKs play crucial roles in various developmental processes. CAM-1, the *Caenorhabditis elegans* ortholog of Ror2, is implicated in cell migration, asymmetric cell division, and axon outgrowth during embryogenesis, and these processes may be either tyrosine kinase-dependent or -independent (14). Previous studies with *Ror2*-deficient mice have further revealed that Ror2 plays crucial roles in the development of the skeletal, genital, and cardiovascular systems (15–17). In humans, *Ror2* is responsible for two heritable skeletal disorders; recessive Robinow syndrome and dominant brachydactyly type B (BDB) (18–23). Interestingly, it has recently been reported that the developmental pathology of $Ror2^{-/-}$ mice can explain many of the developmental malformations found in patients with Robinow syndrome (24).

We have recently shown that Ror2 associates with the melanoma-associated antigen family protein, Dlxin-1, which exhibits a similar developmental expression pattern with Ror2 and is known to bind to the homeodomain proteins Msx2 and Dlx5. Ror2 appears to affect transcriptional functions of Msx2 and Dlx5 by regulating intracellular distribution of Dlxin-1 in a tyrosine kinase-independent manner (25). Furthermore, our

* This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, a Research Grant for Pediatric Research from the Ministry of Health and Welfare of Japan, and by funds from the Yamanouchi Foundation for Research on Metabolic Disorders, the Sumitomo Foundation, Nippon Boehringer Ingelheim, Co., Ltd., and Daiichi Pharmaceutical Co., Ltd. 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.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental figures.

§ These two authors contributed equally to this work.

¶ To whom correspondence may be addressed: Dept. of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5560; Fax: 81-78-382-5579; E-mail: kani@med.kobe-u.ac.jp.

‡‡ To whom correspondence may be addressed: Dept. of Genome Sciences, Faculty of Medical Sciences, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Tel.: 81-78-382-5560; Fax: 81-78-382-5579; E-mail: minami@kobe-u.ac.jp.

[1] The abbreviations used are: RTK, receptor tyrosine kinase; CKIε, casein kinase Iε; GRK2, G protein-coupled receptor kinase 2; BDB, brachydactyly type B; GST, glutathione *S*-transferase; HA, hemagglutinin; WT, wild-type; GMCSF, granulocyte macrophage colony-stimulating factor; WCL, whole cell lysate.

recent genetic and biochemical analyses have indicated that Ror2 interacts with Wnt5a both physically and functionally to activate the noncanonical Wnt5a/JNK pathway in a tyrosine kinase-independent manner (16). In *Xenopus*, Xror2, a putative *Xenopus* ortholog of Ror2, has also been shown to interact with *Xenopus* Wnts and to modulate convergent extension movements of axial mesoderm and neuroectoderm by modulating the planar cell polarity pathway of Wnt signaling in a tyrosine kinase-independent manner (26). However, nothing is known about the molecular mechanisms underlying Ror2 tyrosine kinase activation and the consequent tyrosine kinase-dependent functions of Ror2.

To gain insights into new functions of Ror2, we performed yeast two-hybrid screening using Ror2 as bait to identify a candidate molecule(s) that interacts with Ror2. From this screen, we identified casein kinase Iε (CKIε), a member of the CKI family of protein serine/threonine kinases, as a molecule that interacts with Ror2. Recently, much attention has been paid to CKIε as a crucial regulator of the canonical Wnt signaling, although its exact role(s) in this regulation remains controversial (27). It has been demonstrated that CKIε can phosphorylate various Wnt signaling mediators, including Dvl (Dishevelled), adenomatous polyposis coli, axin, and β-catenin, thereby contributing to the regulation of the canonical Wnt pathway (28–33). Here we show that Ror2 associates with and is phosphorylated on serine/threonine residues by CKIε when expressed in mammalian cells. Interestingly, serine/threonine phosphorylation of Ror2 by CKIε is followed by the autophosphorylation of Ror2 tyrosine residue(s) within its cytoplasmic Pro-rich domain. Moreover, Ror2 associates with G protein-coupled receptor kinase 2 (GRK2) and tyrosine phosphorylates it following activation of Ror2 by CKIε. These results indicate that the tyrosine kinase activity and tyrosine autophosphorylation of Ror2 can be positively regulated by CKIε. We further provide evidence indicating that tyrosine autophosphorylation of Ror2 is required for activation of Ror2 tyrosine kinase.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Wild-type and mutant cDNAs were constructed in the mammalian expression vector pcDNA3 (Invitrogen). Expression vectors encoding the FLAG-tagged Ror proteins were constructed as described previously (25). The Ror2 mutant constructs (ΔC, BDB, RS, Tc, Δ883, Δpro, ΔS/T1, ΔS/T2, and ΔS/T1,2) were generated by deleting amino acids 788–944, 749–944, 502–944, 434–944, 883–944, 783–859, 744–782, 860–882, 744–782 and 860–882, respectively, in the C-terminal region of Ror2. An expression vector encoding a kinase-dead mutant of Ror2 was constructed by replacing lysine 507, crucial for ATP binding, with arginine. Ror2 mutants bearing substitutions of serine and threonine with alanines, Ror2 13S/TA (S860A, S861A, S864A, S866A, S868A, S870A, S879A, and S882A; T869A, T871A, T875A, T876A, and T881A) were constructed by site-directed mutagenesis. Ror2 mutants bearing substitutions of tyrosines with phenylalanines, Ror2 4YF (Y641F, Y645F, Y646F, and Y722F) and Ror2 5YF (Y818F, Y824F, Y830F, Y833F, and Y838F), were also constructed by site-directed mutagenesis. The cDNA fragment corresponding to CKIε was obtained by PCR and inserted into pcDNA3. The expression vector pcDNA-HA-CKIε DK, encoding a kinase-dead mutant of CKIε, was constructed by replacing lysine 38, crucial for ATP binding, with arginine. The plasmids encoding the GST fusion proteins, GST-CKIε WT and GST-CKIε DK, were constructed using the pGEX plasmids (Amersham Biosciences). Bovine GRK2 cDNA (kindly provided by Dr. Haga, Gakushuin University and Dr. Lefkowitz, Duke University) was subcloned into pcDNA3 together with the influenza hemagglutinin (HA) protein epitope tag at its C terminus (pcDNA-GRK2-HA).

Antibodies, Cells, and Transfection—Rabbit polyclonal anti-mouse Ror2 antibody was raised against GST mouse Ror2 (amino acids 726–945). The mouse monoclonal antibodies M2 (Sigma) and 12CA5 (Roche Applied Science) recognize the FLAG peptide and human influenza HA protein peptide sequence. Mouse monoclonal anti-CKIε antibody was purchased from Transduction Laboratories. The mouse monoclonal anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from Cell Signaling and Upstate Biotechnology, Inc., respectively. Rabbit polyclonal anti-phosphoserine and anti-phosphothreonine antibodies were from Zymed Laboratories and Cell Signaling, respectively. HEK293T (293T) and NIH3T3 (3T3) cells were maintained in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% (v/v) fetal calf serum. Transient cDNA transfection was performed using the calcium phosphate method (12).

Immunoprecipitation and Immunoblotting—The cells were solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and the cell lysates were prepared by centrifugation at 12,000 × *g* for 15 min. The cell lysates were precleared for 1 h at 4 °C with protein A-Sepharose (Amersham Biosciences). The precleared supernatants were then immunoprecipitated with anti-FLAG or anti-HA antibody conjugated to protein A-Sepharose beads for 2 h at 4 °C. The immunoprecipitates were washed five times with 1 ml of the above lysis buffer and eluted with Laemmli sample buffer. Immunoprecipitates or whole cell lysates were separated by SDS-PAGE (9% PAGE) and transferred to polyvinylidene difluoride membrane filters (Immobilon, Millipore). The membranes were immunoblotted with the respective antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated anti-mouse IgG antibodies using chemiluminescence reagents (Western Lightning; PerkinElmer Life Sciences) as described previously (12).

Expression and Purification of GST Fusion Proteins—The GST fusion proteins, GST-CKIε WT and GST-CKIε DK, expressed in *Escherichia coli* DH5α were extracted with phosphate-buffered saline containing 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and were isolated with glutathione-Sepharose beads (Amersham Biosciences). Fusion proteins were then eluted from beads by 25 mM glutathione (reduced), followed by dialysis prior to use in kinase assays.

In Vitro Kinase Assay—For *in vitro* kinase assay, 293T cells were solubilized 60 h after transfection, and Ror2 WT-FLAG or Ror2 DK-FLAG proteins were immunoprecipitated as described above. Precipitates were washed five times with lysis buffer and resuspended in 50 μl kinase buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 40 μM ATP. The *in vitro* kinase reaction was initiated by the addition of purified GST-CKIε WT or GST-CKIε DK and allowed to incubate for 30 min at 30 °C. The reaction was terminated by the addition of Laemmli sample buffer, and the samples were separated by SDS-PAGE (10% PAGE) and transferred to polyvinylidene difluoride membrane filters, followed by immunoblot analysis with anti-phospho-serine/threonine antibodies.

In Situ Hybridization—*In situ* hybridization analyses were performed essentially as described previously (34). The 0.86-kb HincII/EcoRI fragment of *Ror2* or the 0.87-kb HincII/ApaI fragment of *GRK2* were utilized as templates to synthesize single strand RNA probes.

RESULTS

Ror2 Associates with CKIε—To identify a Ror2-interacting protein(s), we performed a yeast two-hybrid screening using the cytoplasmic region of Ror2 as bait (25). From this screen we identified a protein serine/threonine kinase, CKIε (data not shown). To determine whether Ror2 associates with CKIε in mammalian cells, FLAG-tagged wild-type Ror2 (Ror2 WT) and HA-tagged CKIε were coexpressed in 293T cells. As shown in Fig. 1*A*, HA-tagged CKIε was coimmunoprecipitated with FLAG-tagged Ror2, indicating that Ror2 associates with CKIε *in vivo*. We also found that endogenous CKIε was detected in anti-Ror2 immunoprecipitates from 3T3 cells, confirming association between endogenous Ror2 and CKIε (Fig. 1*B*).

Next, to identify a cytoplasmic domain(s) within Ror2 required for its association with CKIε, we generated a series of truncated mutants of Ror2 (Fig. 1*C*) and evaluated their abilities to associate with CKIε in 293T cells. As shown in Fig. 1*D*, the Ror2 mutants Ror2 ΔC and Ror2 BDB (Fig. 1*C*) exhibited apparently decreased levels of CKIε binding compared with the Ror2 WT. To map more precisely an association domain within the C-terminal portion of Ror2, we generated additional deletion mutants of Ror2 (Δ883, ΔS/T2, Δpro, ΔS/T1, and ΔS/T1,2) (Fig. 1*C*) and tested their abilities to associate with CKIε. As shown in Fig. 1*E*, among them only the Ror2 Δpro exhibited

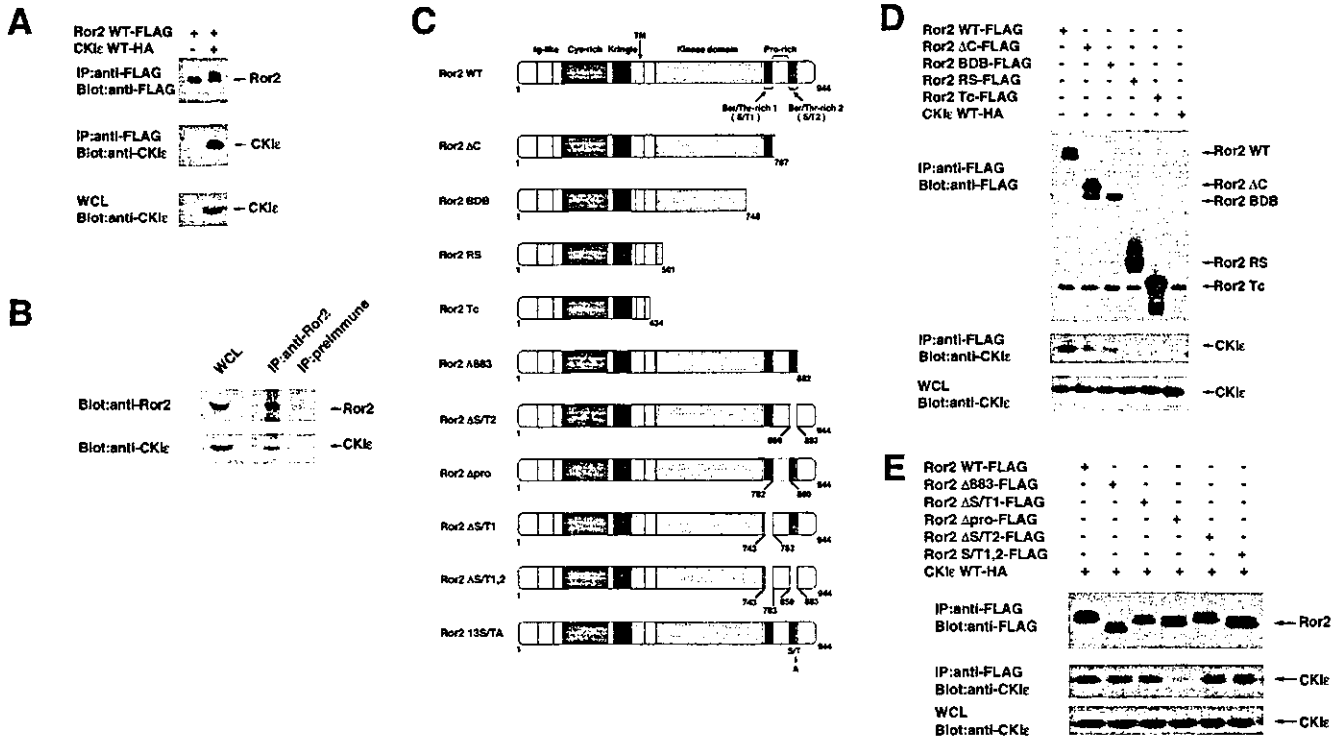


FIG. 1. **Ror2 associates with CKIε *in vivo*.** *A*, association of Ror2 with CKIε in 293T cells. FLAG-tagged Ror2 protein was expressed transiently in 293T cells with or without HA-tagged CKIε protein, as shown in the panel. Whole cell lysates (WCLs) or anti-FLAG immunoprecipitates were analyzed by anti-CKIε or anti-FLAG immunoblotting. *B*, association of endogenous Ror2 with CKIε in NIH3T3 cells. WCLs or immunoprecipitates with anti-Ror2 or control (preimmune serum) antibodies were prepared from 3T3 cells and were analyzed by anti-Ror2 (*upper panels*) or anti-CKIε (*lower panels*) immunoblotting analyses, as shown in the panel. *C*, schematic representations of the WT and a series of deletion mutant forms of Ror2. The *numbers* indicate the positions of the amino acid residues and the putative functional domains within Ror2 are labeled. Serines (860, 861, 864, 866, 868, 870, 879, and 882) and threonines (869, 871, 875, 876, and 881) were replaced with alanines in the 13S/TA mutant. *D* and *E*, determination of a domain(s) within Ror2 protein that is required for the interaction with CKIε. WCLs were prepared from 293T cells expressing FLAG-tagged WT or the respective mutants of Ror2 proteins along with HA-tagged CKIε protein, as shown in the panel. The association of the CKIε with WT and the respective mutant Ror2s were examined as described for *A*.

drastically decreased levels of CKIε binding. The result indicates that the proline-rich domain of Ror2 is required critically for association with CKIε. On the other hand, the Ror2 RS and Ror2 Tc (Fig. 1*C*), both lacking the tyrosine kinase domain, failed to associate with CKIε, suggesting that the tyrosine kinase domain of Ror2 is also required for association (Fig. 1*D*). Because we have previously shown that Ror2 associates with Dlxin-1 (NRAGE) via the proline-rich domain of Ror2 (25), we also examined whether or not association of CKIε and Dlxin-1 with Ror2 is competitive. It was found that association of CKIε with Ror2 was unaffected by ectopic coexpression of Dlxin-1 vice versa (see first supplemental figure).

Serine/Threonine Phosphorylation of Ror2 by CKIε—We next examined whether or not Ror2 is phosphorylated on serine/threonine residues by CKIε in mammalian cells. To this end, FLAG-tagged Ror2 was expressed along with either HA-tagged wild-type (WT) or kinase inactive mutant (DK) of CKIε in 293T cells, and serine/threonine phosphorylation of Ror2 was examined by anti-FLAG immunoprecipitation followed by anti-phosphoserine/threonine immunoblotting (see "Experimental Procedures"). As shown in Fig. 2*A*, Ror2 was phosphorylated on serine/threonine residues in cells coexpressing CKIε WT but not CKIε DK, indicating that the kinase activity of CKIε is required for serine/threonine phosphorylation of Ror2. We also examined whether Dlxin-1 is phosphorylated on serine/threonine residues by CKIε in the presence of Ror2. It appeared that Dlxin-1 was not phosphorylated by CKIε (see second supplemental figure), suggesting that Ror2 may transduce signals via CKIε and Dlxin-1 separately.

To test whether CKIε could phosphorylate Ror2 directly, GST-CKIε WT and GST-CKIε DK proteins purified from *E. coli* (see "Experimental Procedures") were subjected to *in vitro* kinase assay using FLAG-tagged WT or kinase-inactive Ror2 mutant (DK) as substrates. As shown in Fig. 2*B*, GST-CKIε WT, but not GST-CKIε DK, could phosphorylate serine/threonine residues within both Ror2 WT and Ror2 DK *in vitro*, supporting the idea that CKIε phosphorylates Ror2 directly.

We also examined the phosphorylation status of the Ror2 ΔS/T1, ΔS/T2, ΔS/T1,2, and Δpro in mammalian cells coexpressing CKIε. The Ror2 ΔS/T2, ΔS/T1,2, and Δpro exhibited a complete loss of serine/threonine phosphorylation of Ror2, whereas the Ror2 ΔS/T1 showed somewhat weak, yet apparent serine/threonine phosphorylation of Ror2, compared with the Ror2 WT (Fig. 2*C*).

Next, we attempted to identify serine/threonine phosphorylation sites within Ror2 by CKIε. Because serine/threonine phosphorylation of Ror2 was found in the Ror2 ΔS/T1 but not Ror2 ΔS/T2 mutants, we generated the Ror2 13S/TA mutant in which all of the serines and threonines in the S/T2 domain of Ror2 were replaced with alanines (Fig. 1*C*). As shown in Fig. 2*C*, the Ror2 13S/TA mutant could associate with CKIε but failed to be phosphorylated by CKIε, indicating that CKIε phosphorylates primarily serine/threonine residues in the S/T2 domain of Ror2.

Tyrosine Autophosphorylation of Ror2 Following Its Serine/Threonine Phosphorylation by CKIε—To better understand the biological consequence of Ror2 phosphorylation by CKIε, we transiently coexpressed FLAG-tagged Ror2 WT with either CKIε WT or CKIε DK in 293T cells. Tyrosine phosphorylation of Ror2 was detected when Ror2 and CKIε WT were coexpressed, but not when Ror2 was expressed alone or coexpressed with CKIε DK (Fig. 3*A*). The results indicate that CKIε kinase

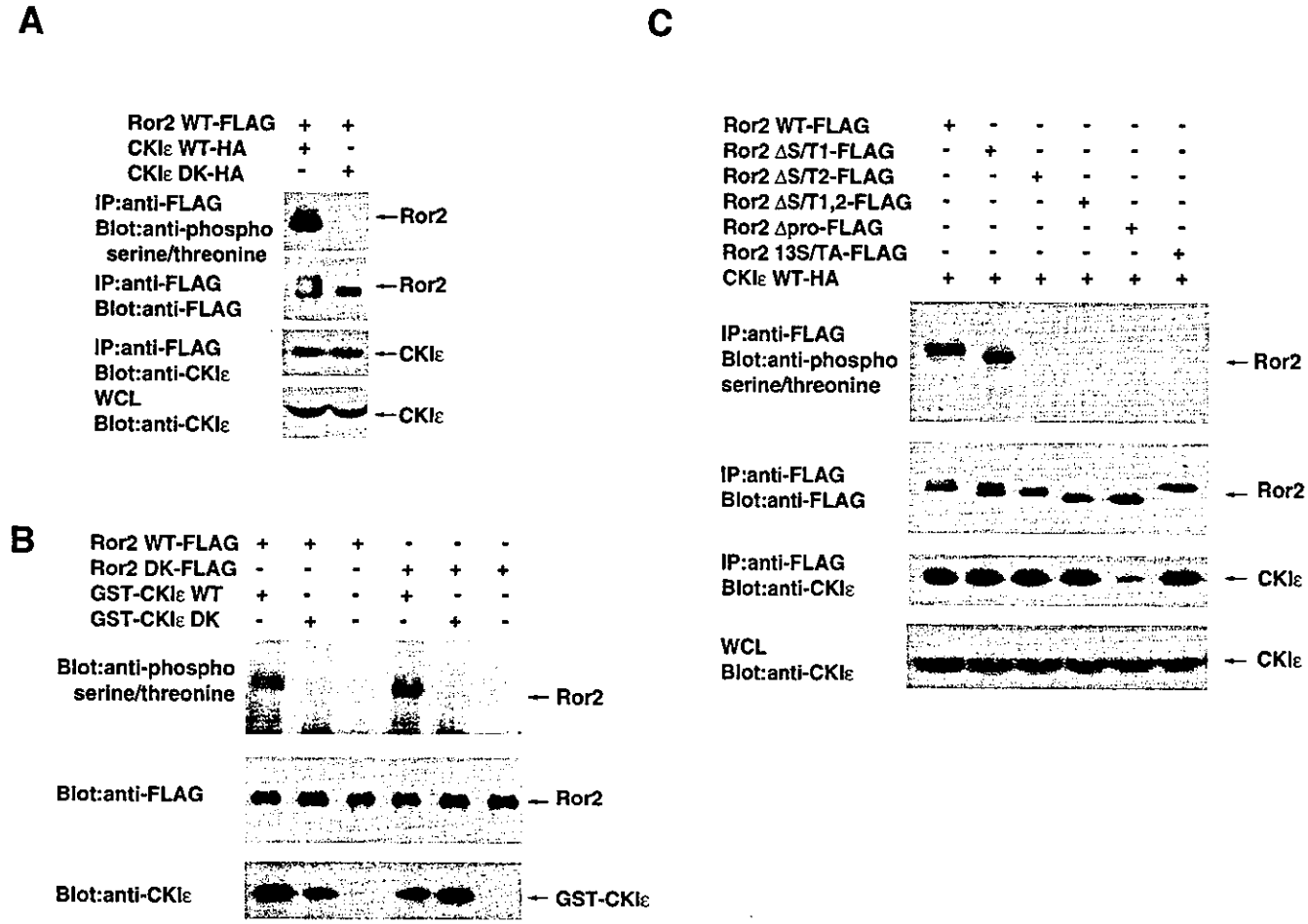


FIG. 2. **Phosphorylation of serine/threonine residues within the serine/threonine rich 2 domain of Ror2 following expression of CKIε.** *A*, serine/threonine phosphorylation of Ror2 following expression of CKIε WT but not CKIε DK. FLAG-tagged Ror2 protein was expressed transiently in 293T cells along with either the WT or kinase inactive mutant (DK) of CKIε proteins. WCLs or anti-FLAG immunoprecipitates (*IP*) from the respective WCLs were analyzed by anti-phosphoserine/threonine (*top panel*), anti-FLAG (*second panel*), or anti-CKIε (*third* and *bottom panels*) immunoblotting. *B*, Ror2 serine/threonine residues are phosphorylated *in vitro* by purified CKIε WT, but not CKIε DK. FLAG-tagged Ror2 WT and Ror2 DK were prepared by overexpressing the respective proteins in 293T cells followed by anti-FLAG immunoprecipitation. *In vitro* kinase assay was performed as described under "Experimental Procedures." *Top*, *middle*, and *bottom panels* indicate anti-phosphoserine/threonine, anti-FLAG, and anti-CKIε immunoblot analyses of kinase reactions, respectively. *C*, serine/threonine phosphorylation within the serine/threonine-rich 2 domain of Ror2 by CKIε. WCLs were prepared from 293T cells expressing FLAG-tagged WT or a series of Ror2 mutant proteins along with HA-tagged CKIε protein, as shown in the panel. Serine/threonine phosphorylation of Ror2 WT and the respective Ror2 mutants were examined as described for *A*.

activity is required for tyrosine phosphorylation of Ror2. Next, FLAG-tagged Ror2 WT or Ror2 DK was expressed along with CKIε WT, and Ror2 tyrosine phosphorylation was evaluated. Tyrosine phosphorylation of Ror2 WT, but not Ror2 DK, was observed under the same experimental setting, although Ror2 DK was also associated with and serine/threonine-phosphorylated by CKIε to similar extents when compared with Ror2 WT (Fig. 3*B*). Thus, CKIε-mediated tyrosine phosphorylation of Ror2 requires the intrinsic tyrosine kinase activity of Ror2.

We further attempted to identify tyrosine phosphorylation sites within Ror2 induced by coexpression of CKIε. We have previously shown that the tyrosine kinase domains of the Ror family RTKs are most similar to those of the neurotrophin receptor Trk family of RTKs and that the four autophosphorylated tyrosine residues found in the activation loops within the tyrosine kinase domains of the Trk family RTKs are also conserved in Ror2 (Tyr^{641}, Tyr^{645}, Tyr^{646}, and Tyr^{722}) (12). We also found that coexpression of CKIε failed to induce tyrosine phosphorylation of Ror2 ΔC (data not shown). The Ror2 ΔC lacks the C-terminal portion of Ror2, which contains six tyrosine residues, including five (Tyr^{818}, Tyr^{824}, Tyr^{830}, Tyr^{833}, and Tyr^{838}) that are found in the Pro-rich domain. Thus, we generated the two Ror2 mutants, 4YF and 5YF (Fig. 3*C* and see "Experimental Procedures"), in which the tyrosines were replaced with phenylalanines. When FLAG-tagged Ror2 4YF and 5YF were expressed in 293T cells along with CKIε, it was found that both the Ror2 4YF and 5YF could associate with CKIε and were phosphorylated on serine/threonine residues to a similar extent as Ror2 WT (Fig. 3*D*). Interestingly, when coexpressed with CKIε, Ror2 4YF but not Ror2 5YF was found to be phosphorylated on tyrosine residues (Fig. 3*D*). This indicates that the sites of tyrosine autophosphorylation are among the five tyrosine residues contained within the Pro-rich domain but not among the four tyrosine residues contained within the tyrosine kinase domain. We then examined whether serine/threonine phosphorylation of Ror2 by CKIε is required for tyrosine autophosphorylation of Ror2. For this purpose, tyrosine phosphorylation status of the 13S/TA was monitored in the presence of CKIε. When Ror2 WT and 13S/TA mutant were expressed in 293T cells along with CKIε, tyrosine autophosphorylation of Ror2 was found in Ror2 WT but not 13S/TA (Fig. 3*E*). This result indicates that serine/threonine phosphorylation of Ror2 by CKIε is required for subsequent tyrosine autophosphorylation of Ror2.

Tyrosine Phosphorylation of GRK2 by Ror2 Following Coexpression of CKIε—The Ror2-interacting proteins, CKIε and

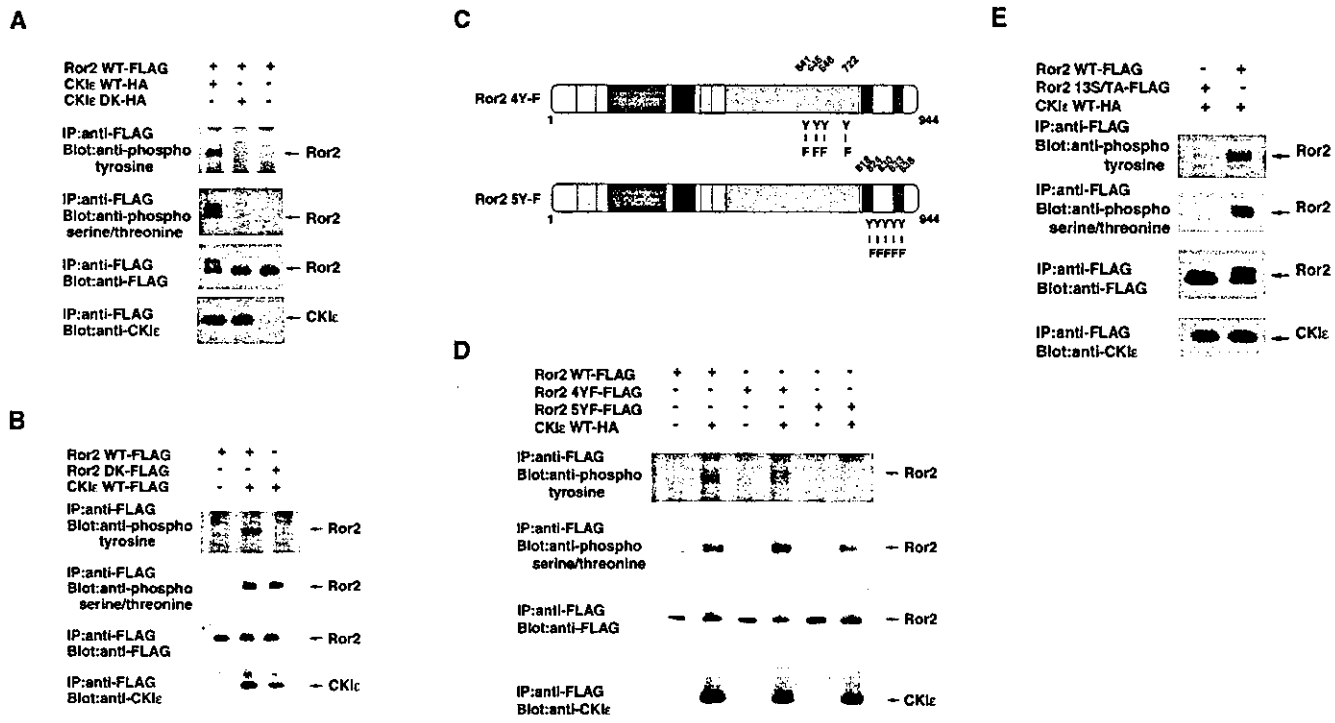


FIG. 3. **Phosphorylation of tyrosine residues within the C-terminal proline-rich domain of Ror2 following expression of CKIε.** *A*, Ror2 is tyrosine-phosphorylated following coexpression of CKIε WT but not CKIε DK. FLAG-tagged Ror2 protein was expressed transiently in 293T cells along with either the WT or a kinase-inactive mutant (DK) CKIε, as shown in the panel. WCLs or anti-FLAG immunoprecipitates (*IP*) from the respective cell lysates were analyzed by anti-phosphotyrosine (*top panel*), anti-phosphoserine/threonine (*second panel*), anti-FLAG (*third panel*), or anti-CKIε (*bottom panel*) immunoblotting. *B*, tyrosine phosphorylation of Ror2 following expression of CKIε requires Ror2 kinase activity. FLAG-tagged WT or DK Ror2 was expressed transiently in 293T cells with or without CKIε protein, as shown in the panel. Tyrosine and serine/threonine phosphorylation of Ror2 were monitored as described in *A*. *C*, schematic representations of the Ror2 mutants (Ror2 4YF and Ror2 5YF) containing different substitutions of tyrosines with phenylalanines. Tyrosines 641, 645, 646, and 722 were replaced with phenylalanines in the 4YF mutant and tyrosines 818, 824, 830, 833, and 838 were replaced with phenylalanines in the 5YF mutant. The *numbers* indicated represent the positions of the respective amino acid residues. *D*, five tyrosine residues within the proline-rich domain of Ror2 are required for tyrosine phosphorylation of Ror2 following expression of CKIε. FLAG-tagged WT or the tyrosine-substituted Ror2 mutants (4YF and 5YF) were expressed transiently in 293T cells with or without CKIε protein, as shown in the panel. Tyrosine and serine/threonine phosphorylation of Ror2 were monitored as described in *A*. *E*, tyrosine phosphorylation of following expression of CKIε requires Ror2 serine/threonine phosphorylation in the serine/threonine rich 2 domain of Ror2. FLAG-tagged WT or Ror2 13S/TA was expressed transiently in 293T cells along with CKIε protein, as shown in the panel. Tyrosine and serine/threonine phosphorylation of Ror2 were monitored as described for *A*.

Dlxin-1 (see Fig. 5*A*), were not tyrosine-phosphorylated by Ror2 when coexpressed with CKIε (data not shown). We therefore searched for other candidate molecule(s) that could be tyrosine-phosphorylated by Ror2 under the same experimental conditions. The gene encoding the GRK2 was reported to exhibit a developmental expression pattern very similar to those of *Ror2* (Fig. 4*A*) and *Dlxin-1* (data not shown), as verified by whole mount *in situ* hybridization analyses on mouse embryos at embryonic day 10.5 (Fig. 4*A*; data not shown), (34, 35, 36). In particular, *Ror2* and *GRK2* exhibited remarkably similar expression patterns in the pharyngeal arches and developing limb buds at embryonic day 10.5 (Fig. 4*A*). We therefore examined whether or not Ror2 could associate with GRK2. As shown in Fig. 4*B*, HA-tagged GRK2 coimmunoprecipitated with FLAG-tagged Ror2, indicating that Ror2 associates with GRK2 *in vivo*. In addition, it was found that the association of Ror2 with GRK2 was unaffected by coexpression of CKIε (data not shown).

To examine whether or not GRK2 or Dlxin-1 could be tyrosine-phosphorylated by Ror2 following coexpression of CKIε, FLAG-tagged Ror2 and HA-tagged CKIε were coexpressed in 293T cells along with HA-tagged GRK2 or Dlxin-1. As shown in Fig. 5*A*, GRK2, but not Dlxin-1, was tyrosine-phosphorylated when Ror2 and CKIε were coexpressed. Under the same experimental conditions, tyrosine phosphorylation of CKIε by Ror2 was not detected (data not shown). These results reveal that coexpression of Ror2 and CKIε leads to the tyrosine phosphorylation of GRK2. Next, we examined whether or not tyrosine phosphorylation of GRK2 correlates with CKIε-induced tyrosine autophosphorylation of Ror2. Consistent with the result shown in Fig. 5*A*, coexpression of Ror2 WT and CKIε WT resulted in the tyrosine phosphorylation of GRK2, whereas Ror2 WT or CKIε WT alone did not (Fig. 5*B*). Furthermore, coexpression of Ror2 WT plus CKIε DK or Ror2 DK plus CKIε WT failed to induce tyrosine phosphorylation of GRK2. These results indicate that tyrosine phosphorylation of Ror2, following coexpression of Ror2 and CKIε, leads to Ror2-mediated tyrosine phosphorylation of GRK2.

Next, we examined whether tyrosine autophosphorylation of Ror2 is required for Ror2-mediated tyrosine phosphorylation of GRK2. As shown in Fig. 5*C*, tyrosine phosphorylation of GRK2 was observed when Ror2 WT or Ror2 4YF, but not Ror2 5YF, was coexpressed with CKIε. This indicates that autophosphorylation of one or more of the five tyrosine residues within the Pro-rich domain of Ror2 is required for tyrosine phosphorylation of GRK2 by Ror2. Finally, we also examined whether serine/threonine phosphorylation of Ror2 by CKIε is indeed required for Ror2-mediated tyrosine phosphorylation of GRK2. As expected, tyrosine phosphorylation of GRK2 was detected in cells expressing Ror2 WT, but not 13S/TA, along with CKIε (Fig. 5*D*). The result indicates that serine/threonine phosphorylation of Ror2 by CKIε is also required for tyrosine phosphorylation of GRK2 by activated Ror2.

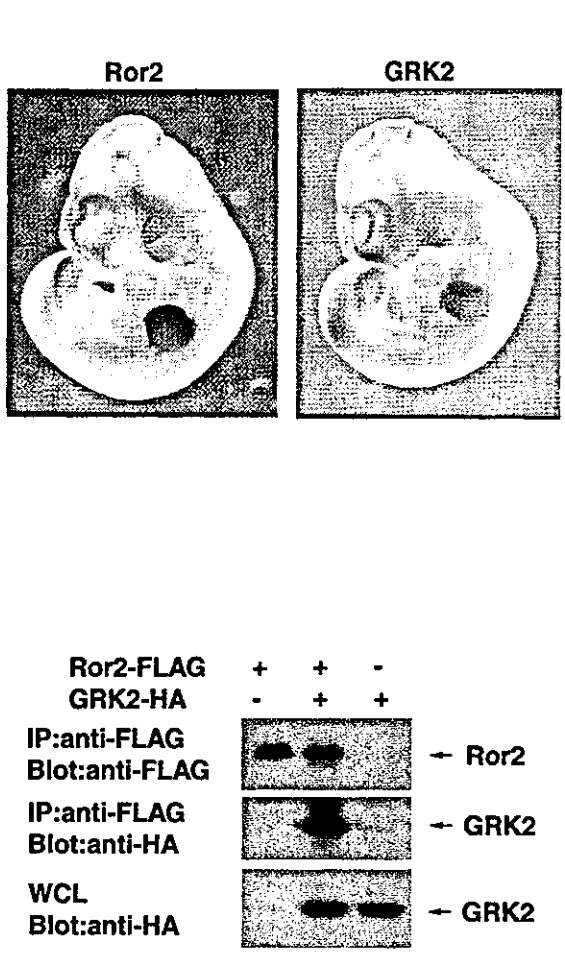


FIG. 4. **Ror2 associates with GRK2 *in vivo*.** *A*, developmental expression patterns of *Ror2* and *GRK2*. *In situ* hybridization of whole mouse embryos (embryonic day 10.5) was performed as described under "Experimental Procedures." *Ror2* (*left panel*) and *GRK2* (*right panel*) were expressed in a similar manner in the pharyngeal arches and developing limbs. *B*, association of Ror2 with GRK2 in 293T cells. FLAG-tagged Ror2 protein (WT) was expressed transiently in 293T cells with or without HA-tagged GRK2 protein, as shown in the panel. WCLs or anti-FLAG immunoprecipitates (*IP*) from the respective WCLs were analyzed by anti-FLAG (*top panel*) or anti-HA immunoblotting (*middle* and *bottom panels*).

DISCUSSION

The receptor tyrosine kinase Ror2 plays important roles in developmental morphogenesis, including the development of skeletal, genital, and cardiorespiratory systems (15, 17). Genetic analyses in nematodes have revealed that CAM-1, the *C. elegans* ortholog of Ror2, possesses both tyrosine kinase-dependent and -independent functions in development (9, 13, 14). However, little is known about the tyrosine kinase-dependent functions of Ror2, in particular, the mechanisms underlying tyrosine kinase activation of Ror2. In fact, Wnt5a stimulation of Ror2, antibody-mediated cross-linking of Ror2, and granulocyte macrophage colony-stimulating factor (GMCSF)-induced dimerization of a chimeric GMCSF/Ror2 receptor (GMCSF receptor extracellular region/Ror2 transmembrane and intracellular regions) fail to induce Ror2 tyrosine autophosphorylation or tyrosine kinase activity (data not shown). Here we show that tyrosine autophosphorylation and tyrosine kinase activation of Ror2 are induced by the cytoplasmic protein serine/threonine kinase CKIε-mediated phosphorylation of Ror2 (Fig. 3).

Our structure-function analyses of Ror2 indicate that Ror2 associates with CKIε primarily via its C-terminal proline-rich domain (Fig. 1, *D* and *E*) and that Ror2 is phosphorylated by CKIε in its C-terminal S/T2 domain (Fig. 2*C*). Because all of the deletional mutants of Ror2 lacking the proline-rich domain, Ror2 ΔC, BDB, RS, Tc (data not shown), and Δpro (Fig. 2*C*), exhibited both drastically decreased levels or complete loss of CKIε binding and of serine/threonine phosphorylation by CKIε (Fig. 2*C*; data not shown), it is likely that association of Ror2 with CKIε is required for its phosphorylation by CKIε. Furthermore, we present evidence indicating that phosphorylation of Ror2 by CKIε is prerequisite to induce autotyrosine phosphorylation and activation of Ror2 (Figs. 3 and 5). In fact, the Ror2 13S/TA mutant, which is assumed to lack serine/threonine phosphorylation sites by CKIε, can associate with CKIε but fail to be autotyrosine-phosphorylated or activated by CKIε (Figs. 2*C*, 3*E*, and 5*D*). It is of importance to determine the pivotal serine/threonine residues within the S/T2 domain of Ror2 that are phosphorylated by CKIε. Ror2 mutants lacking the C-terminal portion (*e.g.* Ror2 ΔC and BDB) are also neither autotyrosine-phosphorylated nor activated by CKIε (data not shown). Interestingly, mutant Ror2 proteins from BDB patients lack this C-terminal portion of Ror2, suggesting that the pathogenesis of BDB may be attributable to a defect in tyrosine autophosphorylation and activation of Ror2 tyrosine kinase activity.

To clarify the mechanism of tyrosine kinase activation of Ror2 following phosphorylation by CKIε, we attempted to identify the sites of tyrosine autophosphorylation within the cytoplasmic region of Ror2. It had been reported that autophosphorylation of the tyrosine residues in the activation loop of the tyrosine kinase domains of the Trk family RTKs is required for activating the Trk family RTKs (38), the closest relatives of Ror family RTKs. However, we mapped the Ror2 tyrosine autophosphorylation sites to the proline-rich domain, not the activation loop (Fig. 3*D*). Further study will be required to identify more precisely the autophosphorylated tyrosine residue(s). In this study, we also identified the cytoplasmic protein serine/threonine kinase GRK2, as a substrate for activated Ror2 (Fig. 5). Interestingly, the Ror2 5YF, but not 4YF, failed to tyrosine phosphorylates GRK2 (Fig. 5*C*), indicating that autophosphorylation of tyrosine residue(s) within the proline-rich domain of Ror2 is required for (full) activation of Ror2 tyrosine kinase. We have previously shown that Ror1, in addition to Ror2, plays an important role in developmental morphogenesis and that most functions of Ror1 during mouse development can be compensated for by Ror2 (37). We found that Ror1 could also associate with CKIε *in vivo* and is phosphorylated on serine/threonine residues by CKIε (data not shown). However, at present tyrosine autophosphorylation and tyrosine kinase activation of Ror1 have not been detected, probably because of a very low level of Ror1 expression compared with Ror2 in our transfection experiments. The results also suggest that the mechanism underlying activation of Ror2 tyrosine kinase may be distinct from those of other Trk family RTKs.

Our results demonstrate that the tyrosine kinase activity of Ror2 is regulated by CKIε. It has been reported that several RTKs can be transphosphorylated by cytoplasmic protein kinases. For example, the insulin receptor is phosphorylated by cAMP-dependent protein kinase (7), protein kinase C (8), and casein kinase 2 (6), and the epidermal growth factor receptor is phosphorylated by cAMP-dependent protein kinase (2), protein kinase C (4, 5), and calmodulin-dependent protein kinase II (3). In these cases, serine/threonine phosphorylation of these RTKs results in the drastic down-regulation of their auto-tyrosine phosphorylation and tyrosine kinase activities. Compared with these RTKs, Ror2 RTK is rather unique in that serine/threonine phosphorylation of Ror2 by CKIε results in the stimulation of its tyrosine autophosphorylation and tyrosine kinase

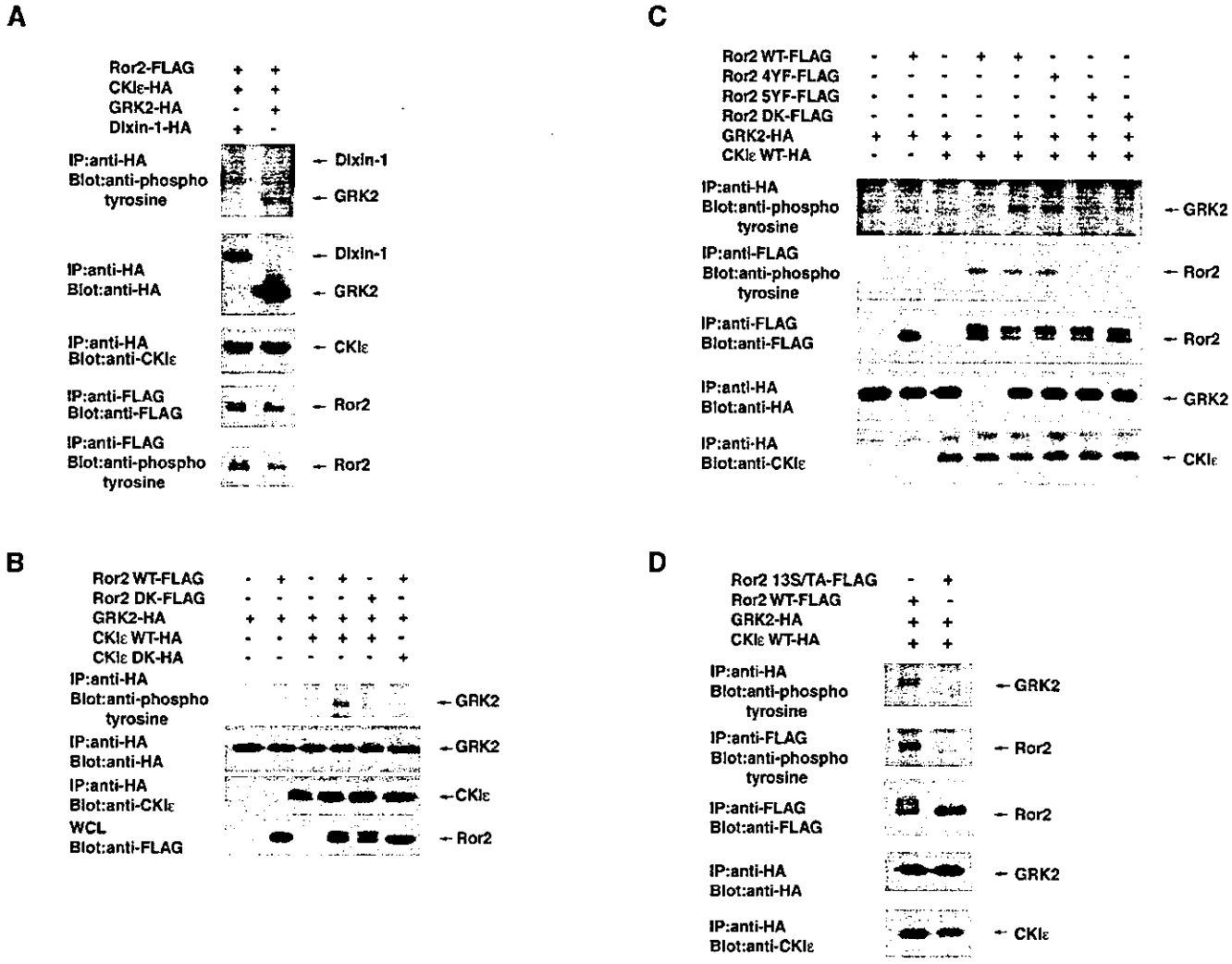


FIG. 5. **Tyrosine phosphorylation of GRK2 by Ror2 following coexpression of CKIε.** *A*, tyrosine phosphorylation of GRK2 following coexpression of Ror2 and CKIε. FLAG-tagged Ror2 and HA-tagged CKIε proteins were coexpressed transiently in 293T cells with or without HA-tagged GRK2 or Dlxin-1, as shown in the panel. Anti-HA or anti-FLAG immunoprecipitates (*IP*) from the respective WCLs were analyzed by anti-phosphotyrosine (*top* and *bottom panels*), anti-HA (*second panel*), anti-CKIε (*third panel*), and anti-FLAG (*fourth panel*) immunoblotting. *B*, tyrosine phosphorylation of GRK2 induced by coexpression of Ror2 and CKIε requires Ror2 and CKIε kinase activities. HA-tagged GRK2 protein was expressed transiently in 293T cells singly or in combination with FLAG-tagged Ror2 proteins (WT or DK) and/or HA-tagged CKIε proteins (WT or DK), as shown in the panel. WCLs and anti-HA immunoprecipitates were analyzed by immunoblotting as described for *A*. *C*, tyrosine residues within the C-terminal proline-rich domain of Ror2 are required for tyrosine phosphorylation of GRK2 by Ror2 in the presence of CKIε. FLAG-tagged WT or the tyrosine-substituted Ror2 mutants (4YF and 5YF) were expressed transiently in 293T cells along with HA-tagged GRK2 and CKIε proteins, as shown in the panel. Anti-HA or anti-FLAG immunoprecipitates from the respective WCLs were analyzed by immunoblotting as described for *A*. *D*, serine/threonine phosphorylation within the serine/threonine rich 2 domain of Ror2 are required for tyrosine phosphorylation of GRK2 by Ror2 in the presence of CKIε. FLAG-tagged Ror2 WT or Ror2 13S/TA were expressed transiently in 293T cells along with both HA-tagged GRK2 and CKIε proteins, as shown in the panel. Anti-HA or anti-FLAG immunoprecipitates from the respective WCLs were analyzed by immunoblotting as described for *A*.

activity. Importantly, it has been reported that CKIε regulates the canonical Wnt pathway by interacting both physically and functionally with various Wnt signal mediators (28–30, 32, 33). Taken together with our findings, we envisage that there may be significant cross-talk between the Ror2 and canonical Wnt signal pathways. Although we have previously shown that Ror2 is also involved in the noncanonical Wnt pathway (16), it is currently unclear whether or not CKIε is likewise involved in noncanonical Wnt signaling. The rat Frizzled-2 (rFz2) is a putative receptor for Wnt5a and has been shown to activate several protein serine/threonine kinases, including protein kinase C, CaMKII, TAK1, and NLK, which mediate the noncanonical Wnt signaling pathway following engagement of upstream receptors (39–42). It would therefore be of interest to examine whether or not these protein kinases may also be able to phosphorylate and regulate Ror2 tyrosine kinase.

GRK2 is known as a key modulator in the internalization of seven transmembrane-spanning G protein-coupled receptors. Following agonist stimulation, GRK2 phosphorylates the most C-terminal cytoplasmic region of G protein-coupled receptors, resulting in the recruitment of β-arrestin and eventual agonist-induced internalization of the G protein-coupled receptors (43). It has previously been shown that the kinase activity and/or stability of GRK2 can also be modulated by tyrosine phosphorylation by the Src protein tyrosine kinase (44–47). Therefore, it is important to determine whether tyrosine phosphorylation of GRK2 by Ror2 may affect GRK2 kinase activity and consequent endocytosis of G protein-coupled receptors. We have shown that Ror2 forms a complex with rFz2 or human Frizzled 5 (hFz5), putative seven transmembrane-spanning type receptors for Wnt5a (16). Furthermore, it has recently been reported that mouse Frizzled 4 (mFz4), another putative seven transmembrane-spanning type receptor for Wnt5a, can be internalized following stimulation with Wnt5a and phorbol myristoyl

acetate, a potent activator of protein kinase C, and that phosphorylated Dvl 2 recruits β-arrestin 2 to mediate internalization of mFz4 (48). The Ror2-associated CKIε may be located in proximity to the putative seven transmembrane-spanning type receptors for Wnt5a (mFz4, rFz2, and hFz5) (16). In addition, CKIε has been shown to phosphorylate the Dvl proteins (30, 32, 49). Therefore, it is conceivable that the Dvl proteins may be phosphorylated by Ror2-associated CKIε, recruit β-arrestin to these Wnt5a receptors, and thereby mediate internalization of these receptors. Alternatively, activated Ror2 tyrosine kinase may phosphorylate and regulate the function of GRK2, which in turn phosphorylates these receptors, resulting in their internalization. Further study will be required to clarify the biological significance of Ror2 tyrosine kinase activation.

Acknowledgments—We thank T. Haga and R. J. Lefkowitz for providing GRK2 cDNA. We also thank M. Lamphier, R. J. Lefkowitz, and K. Matsumoto for a critical reading of the manuscript.

REFERENCES

1. Schlessinger, J. (2000) *Cell* **103,** 211–225
2. Barbier, A. J., Poppleton, H. M., Yigzaw, Y., Mullenix, J. B., Wiepz, G. J., Bertics, P. J., and Patel, T. B. (1999) *J. Biol. Chem.* **274,** 14067–14073
3. Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992) *J. Biol. Chem.* **267,** 1129–1140
4. Davis, R. J. (1988) *J. Biol. Chem.* **263,** 9462–9469
5. Hunter, T., Ling, N., and Cooper, J. A. (1984) *Nature* **311,** 480–483
6. Grande, J., Perez, M., and Itarte, E. (1988) *FEBS Lett.* **232,** 130–134
7. Roth, R. A., and Beaudoin, J. (1987) *Diabetes* **36,** 123–126
8. Takayama, S., White, M. F., and Kahn, C. R. (1988) *J. Biol. Chem.* **263,** 3440–3447
9. Forrester, W. C. (2002) *Cell Mol. Life Sci.* **59,** 83–96
10. Masiakowski, P., and Carroll, R. D. (1992) *J. Biol. Chem.* **267,** 26181–26190
11. Masiakowski, P., and Yancopoulos, G. D. (1998) *Curr. Biol.* **8,** R407
12. Oishi, I., Takeuchi, S., Hashimoto, R., Nagabukuro, A., Ueda, T., Liu, Z.-J., Hatta, T., Akira, S., Matsuda, Y., Yamamura, H., Otani, H., and Minami, Y. (1999) *Genes Cells* **4,** 41–56
13. Yoda, A., Oishi, I., and Minami, Y. (2003) *J. Recept. Signal Transduct. Res.* **23,** 1–15
14. Forrester, W. C., Dell, M., Perens, E., and Garriga, G. (1999) *Nature* **400,** 881–885
15. DeChiara, T. M., Kimble, R. B., Poueymirou, W. T., Rojas, J., Masiakowski, P., Valenzuela, D. M., and Yancopoulos, G. D. (2000) *Nat. Genet.* **24,** 271–274
16. Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G. C., Mundlos, S., Shibuya, H., Takada, S., and Minami, Y. (2003) *Genes Cells* **8,** 645–654
17. Takeuchi, S., Takeda, K., Oishi, I., Nomi, M., Ikeya, M., Itoh, K., Tamura, S., Ueda, T., Hatta, T., Otani, H., Terashima, T., Takada, S., Yamamura, H., Akira, S., and Minami, Y. (2000) *Genes Cells* **5,** 71–78
18. Afzal, A. R., and Jeffery, S. (2003) *Hum. Mutat.* **22,** 1–11
19. Afzal, A. R., Rajab, A., Fenske, C. D., Oldridge, M., Elanko, N., Ternes-Pereira, E., Tuysuz, B., Murday, V. A., Patton, M. A., Wilkie, A. O. M., and Jeffery, S. (2000) *Nat. Genet.* **25,** 419–422
20. Oldridge, M., Fortuna, A. M., Maringa, M., Propping, P., Mansour, S., Pollitt, C., DeChiara, T. M., Kimble, R. B., Valenzuela, D. M., Yancopoulos, G. D., and Wilkie, A. O. M. (2000) *Nat. Genet.* **24,** 275–278
21. Patton, M. A., and Afzal, A. R. (2002) *J. Med. Genet.* **39,** 305–310
22. Schwabe, G. C., Tinschert, S., Buschow, C., Meinecke, P., Wolff, G., Gillessen-Kaesbach, G., Oldridge, M., Wilkie, A. O. M., Komec, R., and Mundlos, S. (2000) *Am. J. Hum. Genet.* **67,** 822–831
23. van Bokhoven, H., Celli, J., Kayserili, H., van Beusekom, E., Balci, S., Brussel, W., Skovby, F., Kerr, B., Percin, E. F., Akarsu, N., and Brunner, H. G. (2000) *Nat. Genet.* **25,** 423–426
24. Schwabe, G. C., Trepczik, B., Suring, K., Brieske, N., Tucker, A., Sharpe, P. T., Minami, Y., and Mundlos, S. (2004) *Dev. Dyn.* **229,** 400–410
25. Matsuda, T., Suzuki, H., Oishi, I., Kani, S., Kuroda, Y., Komori, T., Sasaki, A., Watanabe, K., and Minami, Y. (2003) *J. Biol. Chem.* **278,** 29057–29064
26. Hikasa, H., Shibata, M., Hiratani, I., and Taira, M. (2002) *Development* **129,** 5227–5239
27. Ding, Y., and Dale, T. (2002) *Trends Biochem. Sci* **27,** 327–329
28. Amit, S., Hatzubai, A., Birman, Y., Andersen, J. S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002) *Genes Dev.* **16,** 1066–1076
29. Gao, Z. H., Seeling, J. M., Hill, V., Yochum, A., and Virshup, D. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1182–1187
30. Kishida, M., Hino, S., Michiue, T., Yamamoto, H., Kishida, S., Fukui, A., Asashima, M., and Kikuchi, A. (2001) *J. Biol. Chem.* **276,** 33147–33155
31. Lee, E., Salic, A., and Kirschner, M. W. (2001) *J. Cell Biol.* **154,** 983–993
32. Peters, J. M., McKay, R. M., McKay, J. P., and Graff, J. M. (1999) *Nature* **401,** 345–350
33. Schwarz-Romond, T., Asbrand, C., Bakkers, J., Kuhl, M., Schaeffer, H. J., Huelsken, J., Behrens, J., Hammerschmidt, M., and Birchmeier, W. (2002) *Genes Dev.* **16,** 2073–2084
34. Matsuda, T., Nomi, M., Ikeya, M., Kani, S., Oishi, I., Terashima, T., Takada, S., and Minami, Y. (2001) *Mech. Dev.* **105,** 153–156
35. Sefton, M., Blanco, M. J., Penela, P., Mayor, F., and Nieto, M. A. (2000) *Mech. Dev.* **98,** 127–131
36. Al-Shawi, R., Ashton, S. V., Underwood, C., and Simons, J. P. (2001) *Dev. Genes Evol.* **211,** 161–171
37. Nomi, M., Oishi, I., Kani, S., Suzuki, H., Matsuda, T., Yoda, A., Kitamura, M., Itoh, K., Takeuchi, S., Takeda, K., Akira, S., Ikeya, M., Takada, S., and Minami, Y. (2001) *Mol. Cell. Biol.* **21,** 8329–8335
38. Cunningham, M. E., Stephens, R. M., Kaplan, D. R., and Greene, L. A. (1997) *J. Biol. Chem.* **272,** 10957–10967
39. Ishitani, T., Kishida, S., Hyodo-Miura, J., Ueno, N., Yasuda, J., Waterman, M., Shibuya, H., Moon, R. T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003) *Mol. Cell. Biol.* **23,** 131–139
40. Kuhl, M., Sheldahl, L. C., Park, M., Miller, J. R., and Moon, R. T. (2000) *Trends Genet.* **16,** 279–283
41. Kuhl, M., Sheldahl, L. C., Malbon, C. C., and Moon, R. T. (2000) *J. Biol. Chem.* **275,** 12701–12711
42. Sheldahl, L. C., Park, M., Malbon, C. C., and Moon, R. T. (1999) *Curr. Biol.* **9,** 695–698
43. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem.* **67,** 653–692
44. Elorza, A., Penela, P., Sarnago, S., and Mayor, F., Jr. (2003) *J. Biol. Chem.* **278,** 29164–29173
45. Fan, G., Shumay, E., Malbon, C. C., and Wang, H. (2001) *J. Biol. Chem.* **276,** 13240–13247
46. Penela, P., Elorza, A., Sarnago, S., and Mayor, F., Jr. (2001) *EMBO J.* **20,** 5129–5138
47. Sarnago, S., Elorza, A., and Mayor, F., Jr. (1999) *J. Biol. Chem.* **274,** 34411–34416
48. Chen, W., ten Berge, D., Brown, J., Ahn, S., Hu, L. A., Miller, W. E., Caron, M. G., Barak, L. S., Nusse, R., and Lefkowitz, R. J. (2003) *Science* **301,** 1391–1394
49. Sakanaka, C., Leong, P., Xu, L., Harrison, S. D., and Williams, L. T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12548–12552