OH (pH 7.5), 60 mM KCl, 6 mM $MgCl_2$, 25 μM $ZnCl_2$, and glycerol at 6 to 8% (v/v) (transcription buffer).
12. The p13-agarose beads (Oncogene Science, Santa Cruz Biotechnology, Santa Cruz, CA) were washed three times in transcription buffer (*11*) before incubation with an equal volume of either the mitotic or interphase egg cytosol extracts (*4*) on a rotator for 1 to 2 hours at 4°C. The activated *cdc2*–cyclin B kinase–glutathione-S-transferase (GST) from the mitotic extract was affinity-purified on glutathione-Sepharose (Pharmacia) as described by Solomon and co-workers (*9*). Binding of protein kinase activity from the mitotic extract to p13-agarose or glutathione-Sepharose was confirmed by phosphorylation of histone H1 with [γ-^{32}P]ATP [C. Smythe and J. W. Newport, *Methods Cell Biol.* **35**, 449 (1991)]. For phosphorylation of transcription factors, one-half volume of packed beads was used per volume of transcription factor fractions in the presence of unlabeled nucleoside triphosphates (as for a transcription reaction). Samples were incubated on a rotator at ambient temperature for 1 hour, and the beads were pelleted in a microfuge. The supernatants were then transferred to fresh tubes for the subsequent assay of the transcription activity of the treated factors.
13. W. G. Dunphy, L. Brizuela, D. Beach, J. Newport, *Cell* **54**, 423 (1988); L. Brizuela, G. Draetta, D. Beach, *EMBO J.* **6**, 3507 (1987); F. Fang and J. Newport, *Cell* **66**, 731 (1991).
14. V. J. Wolf and J. M. Gottesfeld, unpublished data.
15. Addition of TFIIIC resulted in a small amount of rescue, but this observation could be the result of the limitation of this factor in the reconstituted system.
16. The phosphocellulose TFIIIB fraction was dialyzed and sequentially fractionated on DEAE-Sephadex and Mono Q FPLC as described (*26*) with the exception that 0.15 M ammonium sulfate was used for elution of TFIIIB from the DEAE resin. DEAE-Sephadex TFIIIB (1 ml; 220 μg) was applied to a 1-ml Mono Q column, and 40 1-ml fractions were collected between 0.1 and 0.6 M KCl in buffer D (*26*). TFIIIB activity was assayed in 25-μl reactions containing TFIIIA, TFIIIC, 4 μl of the PC-C fraction (12 μg of protein) as a source of Pol III, and other components as in (*11*). The PC-C fraction was used as a source of polymerase because Pol III is separated from TFIIIB by DEAE-Sephadex chromatography. For rescue assays, the complete transcription mixture contained the components listed above and 2.5 μl of DEAE-Sephadex TFIIIB (0.7 μg of protein).
17. R. J. White, S. P. Jackson, P. W. Rigby, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1949 (1992).
18. L. Comai, N. Tanese, R. Tjian, *Cell* **68**, 965 (1992); B. P. Cormack and K. Struhl, *ibid.* **69**, 685 (1992); M. C. Schultz, R. H. Reeder, S. Hahn, *ibid.*, p. 697.
19. S. M. Lobo, M. Tanaka, M. L. Sullivan, N. Hernandez, *ibid.* **71**, 1029 (1992); A. K. P. Taggart, T. S. Fisher, B. F. Pugh, *ibid.*, p. 1015; R. J. White and S. P. Jackson, *ibid.*, p. 1041; G. A. Kassavetis *et al.*, *ibid.*, p. 1055.
20. Ligated multimers of a 26–base pair double-stranded oligonucleotide corresponding in sequence to the adenovirus major late promoter TATA box (*17*) or a control 24–base pair oligonucleotide containing the consensus Pol III B-block sequence [H. J. Keller, P. J. Romaniuk, J. M. Gottesfeld, *J. Biol. Chem.* **267**, 18190 (1992)] were coupled to cyanogen bromide–activated Sepharose 6B-Cl as described by the supplier (Sigma). Before use, the resin was washed several times with transcription buffer containing 1 mM dithiothreitol and 1 mM $MgCl_2$. Equal volumes of either the mitotic or interphase extracts and packed DNA-Sepharose were mixed in Eppendorf tubes on a rotator for 1 hour, and unbound proteins were removed by several washes with the same buffer. Bound proteins were eluted with this buffer containing 0.5 M KCl, and we reduced the KCl concentration to 0.1 M by dialysis for 6 to 14 hours at 4°C using a Pierce (Rockford, IL) microdialyzer. The final concentration of TATA-binding proteins from either extract was approximately 100 ng/μl; this concentration represents <0.5% of the starting cytosol protein. The TATA-Sepharose–binding fraction from interphase extracts contained TFIIIB activity (as detected in a complementation assay with purified TFIIIA and the PC-C fraction), but had no transcriptional activity either alone or in combination with purified TFIIIA and TFIIIC, indicating that Pol III is absent from the TATA-binding fraction.
21. Agarose beads coupled with alkaline phosphatase (6.2 units per milligram of beads; Sigma) were washed three times with transcription buffer (*11*), and equal volumes of packed beads and transcription factors or extracts were incubated on a rotator for 1 hour at ambient temperature. The beads were pelleted in a microfuge, and the supernatants were then tested for transcriptional activity.
22. Protein phosphorylation experiments suggest that TBP itself is not a substrate for direct phosphorylation by the *cdc2*–cyclin B kinase (*14*); no protein of the expected molecular mass of *Xenopus* TBP [33 kD; S. Hashimoto *et al.*, *Nucleic Acids Res.* **20**, 3788 (1992)] is phosphorylated in the TFIIIB-TATA–binding fractions. Furthermore, the deduced amino acid sequence of *Xenopus* TBP does not contain the consensus sequence for phosphorylation by *cdc2* kinase, and recombinant *Xenopus* TBP is not a substrate for phosphorylation by the purified kinase (A. Leresche and J. M. Gottesfeld, unpublished data). In contrast, several other polypeptides in the TFIIIB-TAF fraction are phosphorylated in vitro by *cdc2* kinase.
23. G. A. Kassavetis, B. R. Braun, L. H. Nguyen, E. P. Geiduschek, *Cell* **60**, 235 (1990).
24. S. Rupert, E. H. Wang, R. Tjian, *Nature* **362**, 175 (1993); K. Hisatake *et al.*, *ibid.*, p. 179; J. A. Inostroza, F. H. Mermelstein, W. S. Lane, D. Reinberg, *Cell* **70**, 477 (1992).
25. T. Tayoda and A. P. Wolffe, *Anal. Biochem.* **203**, 340 (1992).
26. K. A. Simmen, J. Bernues, J. D. Lewis, I. W. Mattaj, *Nucleic Acids Res.* **20**, 5889 (1992).
27. Plasmids containing Pol III–transcribed sequences from *Xenopus laevis* have been described: the somatic-type 5*S* RNA gene [pXIs 11; R. C. Peterson, J. L. Doering, D. D. Brown, *Cell* **20**, 131 (1980)] and the tRNA gene for TyrD [F. Stutz, E. Gouillaud, S. G. Clarkson, *Genes Dev.* **3**, 1190 (1989)].
28. We thank K. Clemens and P. Zhang for help with FPLC and S. Sharp for discussions. Supported by grants from NIH to J.M.G. (GM26453) and to D.J.F. (GM33279). D.J.F. also received support from the Pew Memorial Trust. P.H. received a grant-in-aid from Sigma Xi.

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Location of cAMP-Dependent Protein Kinase Type I with the TCR-CD3 Complex

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Selective activation of cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase type I (cAKI), but not type II, is sufficient to mediate inhibition of T cell replication induced through the antigen-specific T cell receptor–CD3 (TCR-CD3) complex. Immunocytochemistry and immunoprecipitation studies of the molecular mechanism by which cAKI inhibits TCR-CD3–dependent T cell replication demonstrated that regulatory subunit Iα, along with its associated kinase activity, translocated to and interacted with the TCR-CD3 complex during T cell activation and capping. Regulatory subunit IIα did not. When stimulated by cAMP, the cAKI localized to the TCR-CD3 complex may release kinase activity that, through phosphorylation, might uncouple the TCR-CD3 complex from intracellular signaling systems.

The demonstration of multiple regulatory (R) subunits of cAMP-dependent protein kinase (cAK) showing cell-specific expression and regulation, as well as distinct intracellular compartmentalization, gave support for the idea that different functions of cAMP may be mediated by specific isozymes of cAK (*1*). In human peripheral blood T lymphocytes, the cAKI holoenzyme [composed of two RIα subunits and two catalytic (C) subunits ($RI\alpha_2C_2$)] is soluble, whereas cAK type II (cAKII) ($RII\alpha_2C_2$) is particulate (*2*). Furthermore, activation of cAKI, but not cAKII, is sufficient to mediate the inhibitory effect of cAMP on TCR-CD3–induced T cell replication (*2*). To investigate the possible mechanism for cAKI-mediated inhibition of TCR-CD3–induced T cell replication, we examined the location of cAKI and cAKII in quiescent cells and in cells stimulated through the TCR-CD3 complex.

The subcellular localization of cAKI and cAKII was assessed with antibodies to RIα (anti-RIα) or RIIα (anti-RIIα) and visualized by indirect immunofluorescence with fluorochrome-labeled secondary antibodies

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(3). The intracellular distribution of cAKI and cAKII was compared with that of the TCR-CD3 complex visualized by antibodies to the CD3ε molecule (anti-CD3). In uncapped human T cells, the TCR-CD3 complex is scattered on the cell surface, although slight patching is observed (Fig. 1A). After permeabilization of the cells with Triton X-100 (0.5%) and staining with anti-RIα and the fluorescein isothiocyanate (FITC)–labeled secondary antibody, RIα was distributed almost homogeneously in the cell cytoplasm (Fig. 1B). In combined images that showed staining for both the TCR-CD3 complex and RIα in the same cell, it appeared that the distribution of these two components was random (Fig. 1C). Similar examination of the subcellular localization of the RIIα subunit in uncapped cells revealed that this R subunit was localized to one distinct spot in the cell (Fig. 1G). When we counterstained with 7-amino-actinomycin D (red) to visualize the nucleus, we found that RIIα was located in close proximity to the cell nucleus. The distinct cellular distribution of RIα and RIIα supports our other studies that demonstrated that more than 75% of cAKI (RIα) is soluble, whereas 90% of cAKII (RIIα) is particulate (2).

We next examined the localization of the TCR-CD3 complex, RIα, and RIIα in human T cells after capping of the TCR-CD3 complex (4, 5). After 60 min of stimulation with anti-CD3 (30 min at 4°C and 30 min at 37°C), the TCR-CD3 complex was either patched or completely capped in most of the cells (>80%) (Fig. 1D). In cells in which the TCR-CD3 complex was patched, immunoreactive RIα was concentrated at the inner surface of the plasma membrane. In fully capped cells, the RIα subunit completely localized to one distinct area of the cell (Fig. 1E). An image overlay of the TCR-CD3 complex and RIα showed that the two components completely co-localized in cells that were fully capped (Fig. 1F). The specificity of TCR-CD3–dependent redistribution of RIα is shown by RIIα staining. Activation through the TCR-CD3 complex was not associated with any change in the localization of immunoreactive RIIα (Fig. 1H), and RIIα did not co-localize with the cap (Fig. 1I).

To determine whether the redistribution of RIα during T cell activation involves a physical interaction with the TCR-CD3 complex, we immunoprecipitated proteins (6) from capped and uncapped T cells with anti-CD3, anti-RIα, and an irrelevant monoclonal antibody (mAb) of the same immunoglobulin G (IgG) subclass (control mAb). The immunoprecipitates formed were photoaffinity-labeled with 8-azido-[^{32}P]cAMP in the absence and presence of excess unlabeled cAMP, and we analyzed them by SDS–polyacrylamide gel electrophoresis (PAGE) to identify precipitated R subunits. The anti-CD3 immunoprecipitates from capped T cells contained a single protein that specifically incorporated 8-azido-[^{32}P]cAMP (Fig. 2A). This protein had an electrophoretic mobility identical to that of human RIα (49 kD). In contrast, similar immunoprecipitates of lysates from uncapped human T cells did not contain proteins that specifically incorporated 8-azido-[^{32}P]cAMP. The anti-RIα also precipitated a protein of 49 kD that incorporated 8-azido-[^{32}P]cAMP. The control mAb did not immunoprecipitate RIα. To assess the specificity of the RIα association with the TCR-CD3 complex, we left the major histocompatibility complex class I (MHC I) molecules uncapped or capped and immunoprecipitated them under conditions identical to those we used with the TCR-CD3 complex. In these experiments, co-immunoprecipitation of RIα was not observed (Fig. 2B).

Next we investigated whether the complete cAKI holoenzyme, and not only RIα, was translocated and associated with the TCR-CD3 complex in activated cells. Immunoprecipitates similar to those described in Fig. 2A were analyzed for specific binding of [^{3}H]cAMP and cAK phosphotransferase activity. In these experiments, the binding activity of R and phosphotransferase activity of C were normalized to the activities measured in the anti-RIα immunoprecipitates, which were arbitrarily set to 100%. The anti-CD3 immunoprecipitates from capped cells contained 70 to 80% of the total [^{3}H]cAMP binding and phosphotransferase activity found in anti-RIα immunoprecipitates (Fig. 2C). In contrast, anti-CD3 immunoprecipitates from uncapped T cells contained <10% of the R and C activities of that measured in anti-RIα immunoprecipitates. Further analysis of the anti-CD3, anti-RIα, and control mAb immunoprecipitates by protein immunoblotting showed that anti-CD3 immunoprecipitates from capped T cells contained

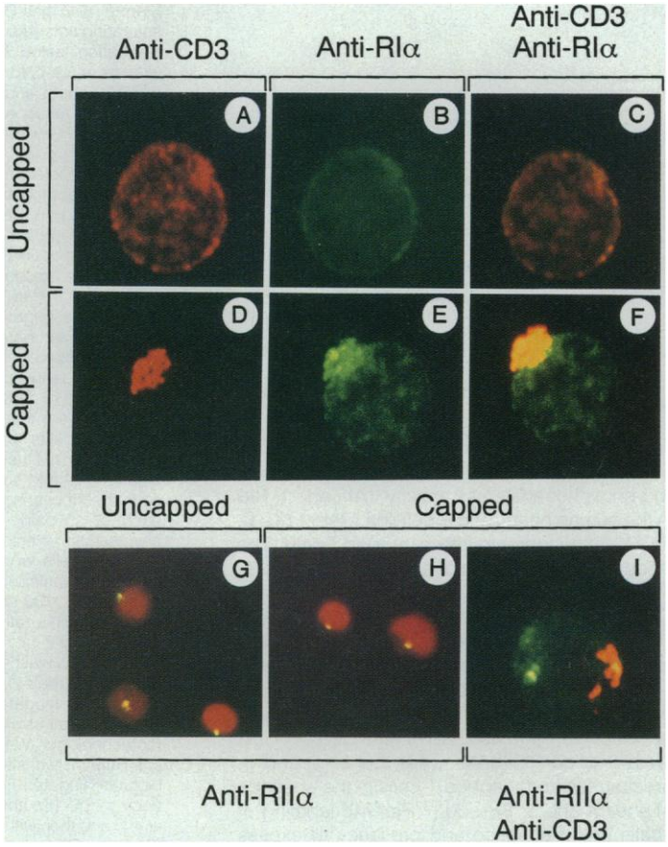

Fig. 1. Localization of the TCR-CD3 complex, RIα, and RIIα in uncapped or TCR-CD3–capped T cells were examined after immunofluorescence labeling in a confocal immunofluorescence microscope. (**A**) RITC (red) fluorescence of uncapped T cells (cells incubated 60 min at 4°C with anti-CD3, 1:100 dilution of ascites, in the presence of 0.1% sodium azide). (**B**) FITC (green) fluorescence of the same cell as shown in (A) after overnight incubation of permeabilized cells with anti-RIα (1:100 dilution of affinity-purified mAb). (**C**) Image overlay of the staining patterns depicted in (A) and (B). Co-localization of TCR-CD3 and RIα gives a yellow fluorescence. (**D**) RITC fluorescence of anti-CD3–capped T cells (incubated with anti-CD3, 1:100 dilution of ascites, for 30 min at 4°C and 30 min at 37°C, in the absence of sodium azide). (**E**) The same cell as shown in (D) is incubated with anti-RIα and FITC-labeled antibody to IgG in the second layer. (**F**) Image overlay of the cell depicted in (D) and (E). (**G**) FITC (green) fluorescence of permeabilized cells incubated with anti-RIIα and counterstained with 7-amino-actinomycin D to visualize the nucleus (red). (**H**) FITC fluorescence (green) in capped T cells incubated with anti-RIIα and counterstained with 7-amino–actinomycin D to visualize the nucleus (red). (**I**) Image overlay of a capped T cell incubated with anti-RIIα and FITC-labeled secondary antibody (green) and with anti-CD3 and RITC-labeled secondary antibody (red).

both immunoreactive RIα and C, but not RIIα or RIβ (Fig. 2D). Complexes immunoprecipitated with anti-CD3 from cell lysates of uncapped cells contained neither the RIα nor the C subunit of cAK (Fig. 2D). An anti-RIα immunoprecipitate of lysates from uncapped T cells contained RIα and C (Fig. 2D). Immunoprecipitation with control mAb did not precipitate any of the cAK subunits. Finally, a protein immunoblot incubated with two different antibodies to the CD3 molecules (clones UCHT1 and SpvT3d) demonstrated that equal amounts of the TCR-CD3 complex were precipitated from both capped and uncapped cells (*7*).

The results described here demonstrate the association of a serine-threonine protein kinase with the TCR-CD3 complex and provide evidence for isozyme-specific effects of cAK in T lymphocytes. Furthermore, TCR-CD3 stimulation is associated with cAMP formation and activation of cAK (*8*). This suggests that relocalization of cAKI in close proximity to the TCR-CD3 complex may establish an inhibitory signaling pathway whereby cAMP can control TCR-CD3–mediated effects by activation of cAKI. Both TCR-CD3–dependent activation of protein kinase C (9) and phosphorylation of phosphoprotein pp100 by TCR-CD3–associated protein tyrosine kinase (PTK) (*10*) are inhibited by cAMP. Furthermore, the human RIα protein (*11*) contains a potential tyrosine phosphorylation site (amino acids 13 to 22) (*12*) and a stretch of prolines and uncharged residues (amino acids 84 to 92) that resembles sequences shown to bind to SH3 domains (*13*). These are putative sites of interaction with TCR-CD3–associated molecules that contain SH3 domains, such as PTKs $p59^{fyn}$ and $p56^{lck}$ or phospholipase C type γ-1. In conclusion, our data suggest a mechanism whereby cAMP, through cAKI-dependent phosphorylation of the TCR-CD3 complex or associated proteins, inhibits antigen-stimulated T cell proliferation.

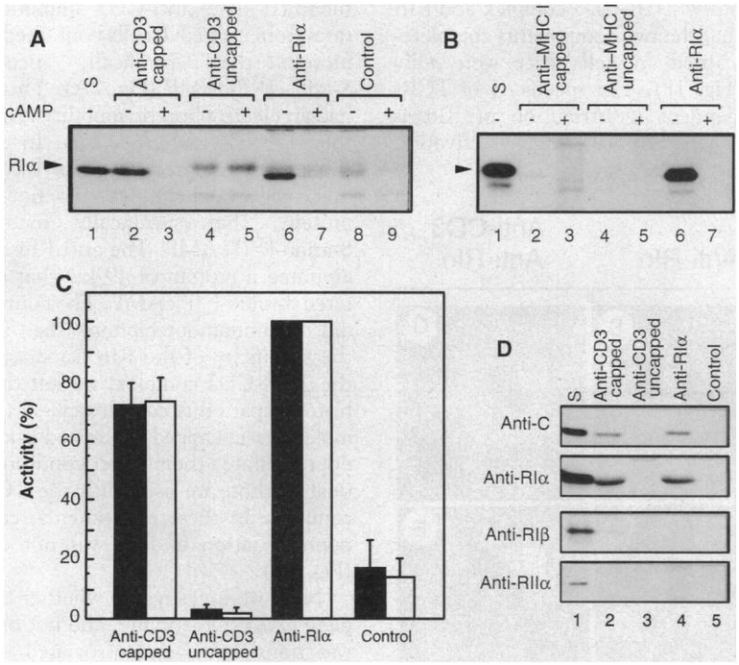

Fig. 2. Detection of R and C subunits in anti-CD3 and anti–MHC I immunoprecipitates of uncapped and capped T cells and analysis of cAMP binding and phosphotransferase activity. (**A**) Lane 1, RIα labeled with 8-azido-[^{32}P]cAMP in a cell extract from the human neoplastic B cell line (Reh) (*14*). S, standard. Lane 2, 8-azido-[^{32}P]cAMP labeling of anti-CD3 immunoprecipitate in capped T cells. The specificity of 8-azido-cAMP incorporation was assessed by the incubation of anti-CD3 immunoprecipitates with excess unlabeled cAMP (100 times the concentration of labeled cAMP) (lane 3). Lanes 4 and 5, 8-azido-[^{32}P]cAMP labeling of anti-CD3 immunoprecipitates from uncapped T cells in the absence (−) and presence (+) of excess unlabeled cAMP, respectively. Lanes 6 and 7, 8-azido-[^{32}P]cAMP labeling of anti-RIα immunoprecipitates in the absence and presence of excess unlabeled cAMP, respectively. Lanes 8 and 9, 8-azido-[^{32}P]cAMP labeling of control mAb (isotype-matched to UCHT1, IgG1κ) immunoprecipitates in the absence and presence of excess unlabeled cAMP, respectively. (**B**) Analysis of the specificity of RIα association with the TCR-CD3 complex. Lane 1, expressed human RIα labeled with 8-azido-[^{32}P]cAMP (*14*). Lanes 2 and 3, 8-azido-[^{32}P]cAMP labeling of anti–MHC I immunoprecipitates from capped T cells in the absence (−) and presence (+) of excess unlabeled cAMP. Lanes 4 and 5, 8-azido-[^{32}P]cAMP labeling of anti–MHC I immunoprecipitates from uncapped T cells in the absence and presence of excess cAMP, respectively. Lanes 6 and 7, 8-azido-[^{32}P]cAMP labeling of anti-RIα immunoprecipitates in the absence and presence of excess unlabeled cAMP, respectively. (**C**) Immunoprecipitates identical to those described in (A) were analyzed for [^{3}H]cAMP binding (solid bars) and cAK phosphotransferase activity (open bars). The R and C activities immunoprecipitated with anti-CD3 (of lysates from capped and uncapped cells) and control mAb are given as percent of activity immunoprecipitated with anti-RIα from untreated cells (mean ± SEM; n = 5). (**D**) Identical immunoprecipitates to those described in (A) and (C) were analyzed for content of the cAK subunits RIα, RIβ, RIIα, and C by protein immunoblotting.

REFERENCES AND NOTES

1. S. M. Lohmann, P. DeCamilli, I. Einig, U. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6723 (1984); S. J. Beebe, P. F. Blachmore, T. D. Chrisman, J. D. Corbin, *Methods Enzymol.* **159**, 118 (1988); M. Lanotte *et al.*, *J. Cell. Physiol.* **146**, 73 (1991); J. D. Scott, *Pharmacol. Ther.* **50**, 123 (1991).
2. B. S. Skålhegg *et al.*, *J. Biol. Chem.* **267**, 15707 (1992).
3. We stained human peripheral blood T lymphocytes (>95% pure) for TCR-CD3 by incubating 1 × 10^7 cells per milliliter of culture medium (RPMI 1640, Gibco, Paisley, Scotland, United Kingdom), supplemented with 5% fetal calf serum (FCS; Sigma), with anti-CD3 (clone UCHT1, IgG1κ) in saturating amounts (1:100 dilution of ascites). The incubation lasted for 30 min on ice. After four washes in ice-cold Hanks' balanced salt solution (HBSS; Gibco) supplemented with 1% FCS, the cells were resuspended in culture medium and stained with rhodamine isothiocyanate (RITC)–conjugated goat antibodies to mouse IgG1 (Southern Biotechnology, Birmingham, AL). We induced capping by raising the temperature to 37°C for 30 min [cells incubated for various time periods (20 to 60 min) revealed maximal capping after 30 min at 37°C]. Uncapped cells were kept on ice in the presence of 0.1% sodium azide. The cells (0.5 × 10^6 to 1 × 10^6) were then plated on poly-L-lysine (Sigma)–coated glass microscopic slides and incubated for 10 min at 4°C. Plated cells were fixed to the glass with 3% (w/v) paraformaldehyde in isotonic phosphate-buffered saline (PBS; pH 7.5) for 7 min at room temperature. The cells were then permeabilized for 10 min at 4°C in a buffer containing 0.5% Triton X-100 (Sigma), 20 mM tris-HCl (pH 7.5), 150 mM sodium chloride, 10 mM sodium fluoride (Sigma), 1 mM sodium orthovanadate (Sigma), and 10 μg/ml each of antipain, pepstatin A, leupeptin, and chymostatin (Peninsula Laboratories, Belmont, CA). The cells were washed with ice-cold PBS before immunolabeling with either anti-RIα (IgG_{2a}, clone 4D7; 1:100 dilution of affinity-purified mAb) (*14*) or anti-RIIα (affinity-purified rabbit antiserum, 1:100 dilution) (*2, 14*) overnight at 4°C. Excess antibody was washed off with ice-cold PBS. Finally, cells incubated with anti-RIα were incubated with FITC-conjugated $F(ab')_2$ fragments of goat antibody to mouse IgG_{2a} (diluted 1:100; Southern Biotechnology). We labeled cells incubated with anti-RIIα in two steps by incubating them with biotin-conjugated $F(ab')_2$ fragments of goat antibody to IgG (Southern Biotechnology) for 30 min at 4°C and then with FITC-conjugated streptavidin (Southern Biotechnology). Stained cells were examined with a Labophot microscope (Nikon) containing an epifluorescence attachment and equipped with a confocal laser scan unit (MRC 600) with a Kr-Ar laser, a K1 double excitation–double dichroic excitation filter block, and a K2 dichroic emission filter block (Bio-Rad, Hertfordshire, United Kingdom). This combination allowed simultaneous detection of FITC and Texas red or RITC fluorescence. A Freeze-Frame unit (Po-

laroid) was attached for photographic documentation of acquired images. A Nikon plan Apo 60 oil immersion objective (NA 1.4) was used for confocal microscopy.
4. R. B. Taylor, W. P. H. Duffus, M. C. Raff, S. de Petris, *Nature* **233**, 225 (1971).
5. A. A. Khan, J. P. Steiner, M. G. Klein, M. F. Schneider, S. H. Snyder, *Science* **257**, 815 (1992).
6. We immunoprecipitated the TCR-CD3 complex and the MHC I from capped T lymphocytes by incubating 2.5 × 10^7 cells with either anti-CD3 (clone UCHT1, 1:10 dilution of ascites) or antibody to MHC I [(anti–MHC I; clone W6/32 (Seralab Laboratories, Sussex, United Kingdom), 1:10 dilution of ascites] for 45 min at 4°C. The cells were then incubated with excess protein A–Sepharose 4B beads coated with rabbit antibody to mouse IgG (Pharmacia) for 60 min at 4°C and centrifuged. Sedimented cells were resuspended in lysis buffer [20 mM tris-HCl (pH 7.5), 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 μg/ml each of antipain, pepstatin A, leupeptin, and chymostatin] containing 1% Brij 96 (Sigma) and incubated for 30 min at 4°C. Lysates were centrifuged at 14,000*g* for 10 min and washed twice in ice-cold lysis buffer. We treated uncapped T lymphocytes (2.5 × 10^7) by lysing the cells in lysis buffer containing 1% Brij 96 and then by immunoprecipitating them with either anti-CD3 (1:10 dilution of ascites), anti–MHC I (1:10 dilution of ascites), anti-RIα (1:10 dilution of ascites), or control mAb (1:10 dilution of ascites) for 60 min at 4°C. The photoaffinity labeling with 8-azido-[^{32}P]cAMP (specific activity, 50 Ci/mmol; ICN, Irvine, CA), the determination of specific [^{3}H]cAMP binding, the determination of cAK phosphotransferase activity, and the protein immunoblot analyses of immunoreactive R and C subunits were done as described [B. S. Skålhegg *et al.*, *J. Biol. Chem.* **267**, 5374 (1992); (*14*)].
7. B. S. Skålhegg *et al.*, unpublished data.
8. J. A. Ledbetter *et al.*, *J. Immunol.* **137**, 3299 (1986); G. Kammer, C. A. Boehm, S. A. Rudolph, L. A. Schultz, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 792 (1988); B. S. Skålhegg, unpublished data.
9. D. J. Park, H. K. Min, S. G. Rhee, *J. Biol. Chem.* **267**, 1496 (1992); R. D. Klausner *et al.*, *ibid.* **262**, 12654 (1987); H. Takayama, G. Tren, M. V. Sitkovsky, *ibid.* **263**, 2330 (1988).
10. E. D. Anastassiou, F. Paliogianni, J. P. Balow, H. Yamada, D. T. Boumpas, *J. Immunol.* **148**, 2845 (1992).
11. M. Sandberg, K. Taskén, O. Øyen, V. Hansson, T. Jahnsen, *Biochem. Biophys. Res. Commun.* **149**, 939 (1987); M. Sandberg, B. Skålhegg, T. Jahnsen, *ibid.* **167**, 323 (1990).
12. T. Patschinsky, T. Hunter, F. S. Esch, J. A. Cooper, B. M. Sefton, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 973 (1982); T. Hunter, *J. Biol. Chem.* **257**, 4843 (1982); J. A. Cooper, F. S. Esch, S. S. Taylor, T. Hunter, *ibid.* **259**, 7835 (1984).
13. P. Cicchetti, B. J. Mayer, G. Thiel, D. Baltimore, *Science* **257**, 803 (1992); H. Yu *et al.*, *ibid.* **258**, 1665 (1992); M. Rozakis-Adcock, R. Fernley, J. Wade, T. Pawson, D. Bowtell, *Nature* **363**, 83 (1993); N. Li *et al.*, *ibid.*, p. 85; G. W. Booker *et al.*, *Cell* **73**, 813 (1993).
14. K. Taskén *et al.*, *J. Biol. Chem.* **268**, 21276 (1993).
15. We thank E. S. Karlstrøm and L. Mossin for technical assistance and P. Beverly (Imperial Cancer Research Fund, London, UK) for clone UCHT1. Supported by the Norwegian Cancer Society, the Norwegian Research Council, Torsted's grant, the Anders Jahre Foundation for the Promotion of Science, and the Nordic Insulin Foundation Committee.

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A Transforming Growth Factor β Type I Receptor That Signals to Activate Gene Expression

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Transforming growth factor beta (TGF-β) is a multifunctional factor that regulates many aspects of cellular functions. TGF-β signals through a heteromeric complex of the type I and type II TGF-β receptors. However, the molecular mechanism of signal transduction by this receptor complex remains unresolved. The type II receptor belongs to a transmembrane receptor serine-threonine kinase family. A new member of this receptor family (R4) was identified and shown to be a functional TGF-β type I receptor on the basis of its ability to restore a TGF-β–induced gene response in mutant cell lines lacking endogenous type I receptor. Both ligand binding and signaling of the R4 protein were dependent on the presence of a functional type II receptor. The type I receptor has an intrinsic serine-threonine kinase activity, which was essential for signal transduction.

The transforming growth factor betas (TGF-βs) are a family of multifunctional cytokines that regulate many aspects of cellular function, including cell proliferation, differentiation, adhesion, and migration (*1*). TGF-β signals through a heteromeric complex between the type I and type II receptors (*2*, *3*). The type II receptor can directly bind ligand, but is incapable of mediating TGF-β responses in the absence of a type I receptor (*3*, *4*). The type II TGF-β receptor has been cloned and is a member of the transmembrane receptor serine-threonine kinase family (*5*). Recently, a murine receptor serine-threonine kinase, Tsk 7L, was concluded to be a type I receptor for both TGF-β and activin because of its biochemical properties (*6*). However, the ability of the Tsk 7L protein to mediate biological responses to TGF-β was not shown.

Because the type II receptors for both TGF-β and activin belong to the same group of receptors (*7*), it was hypothesized that there may be a family of such receptors for the ligands of the TGF-β superfamily. To explore this possibility, we used a polymerase chain reaction cloning strategy to isolate other members of this receptor family. Four putative receptor serine-threonine kinases (R1 through R4) were isolated from the urogenital ridge of 14.5- to 15-day fetal Sprague-Dawley rats (*8*) (Fig. 1). Sequence comparison revealed that the R1 clone represents the rat homologue of Tsk 7L. Because clones R1 through R4 have a high degree of sequence similarity, we investigated the functional properties of all four proteins.

Mink lung epithelial (MvlLu) cells are highly responsive to the effects of TGF-β. Through chemical mutagenesis, several classes of TGF-β–resistant MvlLu cell lines have been generated (*4*, *9*). Mutants of MvlLu cells defective in either the type I (R mutants) or type II (DR mutants) TGF-β receptors lack TGF-β–induced gene expression and TGF-β–induced growth inhibition (*3*, *4*, *9*). A TGF-β reporter construct (p3TP-Lux) containing a luciferase gene

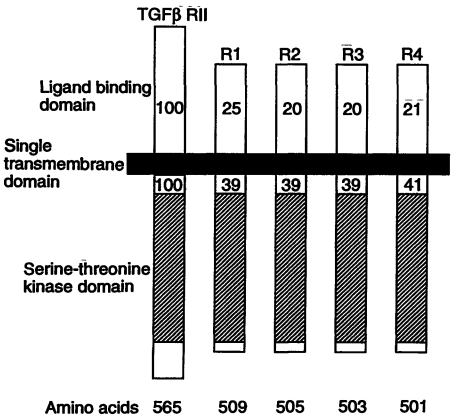

Fig. 1. Schematic diagram comparing the amino acid sequences of R1 through R4 to the TGF-β type II receptor. The percent amino acid similarity of the extracellular and kinase domains of each clone as compared to the type II receptor sequence is indicated. The sequence similarities were generated by the GAP program of the Genetics Computer Group. The nucleotide sequence of the R4 clone has been deposited to GenBank (accession number L26110). The number of amino acids in each protein is indicated.

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