shorter half-lives.

Transcriptional readouts are versatile screens for drug candidates, because most signaling pathways result in expression or repression of specific response elements and genes. Steps in disease progression likewise involve modulation of expression patterns, which can be monitored by transcriptional reporters even if the disease-causing mechanisms are not yet understood. The selectivity and sensitivity of the β-lactamase transcriptional readout as a screen for drugs was assessed with a library of known pharmacophores (Microsource Discovery Systems, Gaylordsville, Connecticut). This library of 480 biologically active compounds was tested in a blind screen for muscarinic agonism and antagonism in 96-well microplates containing the C2 cell line. Known agonists (acetylcholine, arecholine, bethanechol, carbachol, methacholine, and pilocarpine) were identified at a test concentration of 5 μM, and no other compounds in this set displayed agonist activity. In the primary screen for antagonist activity, 41 compounds were active (>80% inhibition of the response induced by 100 μM carbachol). Rescreening in a cell line lacking the M_1 receptor distinguished the 13 true receptor antagonists from 28 compounds that inhibited signal transduction further downstream.

A concentration of about 50 β-lactamase molecules per cell, or 60 pM in a 1.4-pl Jurkat cell, produces in 16 hours a blue-green ratio well above that of nontransfected cells, as apparent by unaided color vision or color film (Fig. 3). By decreasing the duration of exposure to substrate, we measured expression levels up to about 20,000 molecules per cell in situ. A yet higher dynamic range was quantifiable in lysates (Fig. 2B). GFP is the only reporter of single-cell gene expression whose assay is less invasive than that of β-lactamase, but because GFP is not catalytic, about 1 μM cytosolic GFP (10^5 to 10^6 molecules per cell) is necessary to show up over autofluorescence background (26). Thus, most applications of GFP in mammalian cells have needed strong constitutive promoters, typically from viruses, rather than weaker promoters responsive to native mammalian signal transduction pathways. However, β-lactamase does not yet supplant GFP as a tag for subcellular localization of cytoplasmic fusion proteins, because CCF2 and its cleavage product diffuse throughout the cytosol. The β-lactamase reporter system will facilitate many applications such as genetically tagging transfected mammalian cells, engineering cell lines with specific expression phenotypes, expression cloning of molecules that can be linked to gene transcription, trapping genes whose expression levels are developmentally or pharmacologically regulated, and identifying natural or pharmaceutical ligands for receptors.

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Dimerization-Induced Inhibition of Receptor Protein Tyrosine Phosphatase Function Through an Inhibitory Wedge

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The function and regulation of the receptorlike transmembrane protein tyrosine phosphatases (RPTPs) are not well understood. Ligand-induced dimerization inhibited the function of the epidermal growth factor receptor (EGFR)–RPTP CD45 chimera (EGFR-CD45) in T cell signal transduction. Properties of mutated EGFR-CD45 chimeras supported a general model for the regulation of RPTPs, derived from the crystal structure of the RPTPα membrane-proximal phosphatase domain. The phosphatase domain apparently forms a symmetrical dimer in which the catalytic site of one molecule is blocked by specific contacts with a wedge from the other.

The RPTPs are a family of signaling molecules whose function and regulation are not well understood (*1*). In T cells, the RPTP CD45 is required for T cell development (*2*) and T cell receptor (TCR) signal transduction (*3–5*), presumably by dephosphorylating the negative regulatory COOH-terminal tyrosine in the Src-family kinase Lck (*6*). A chimeric EGFR-CD45 molecule restores TCR-mediated signal transduction in a CD45-deficient T cell line; furthermore, treatment of these cells with EGF blocks TCR-mediated signaling, which suggests that CD45 is negatively regulated by ligand-induced dimerization (*7*). A possible explanation for this negative regulation comes from the crystal structure of the membrane-proximal phosphatase domain of the RPTP, RPTPα, which revealed a putative inhibitory wedge in symmetrical dimers (*8*). Two acidic residues found in this wedge are strongly conserved among the membrane-proximal phosphatase domains of RPTPs (*8*). Thus, ligand induced-dimerization may result in inhibition of phosphatase activity, and consequently of signaling function, through specific interactions between

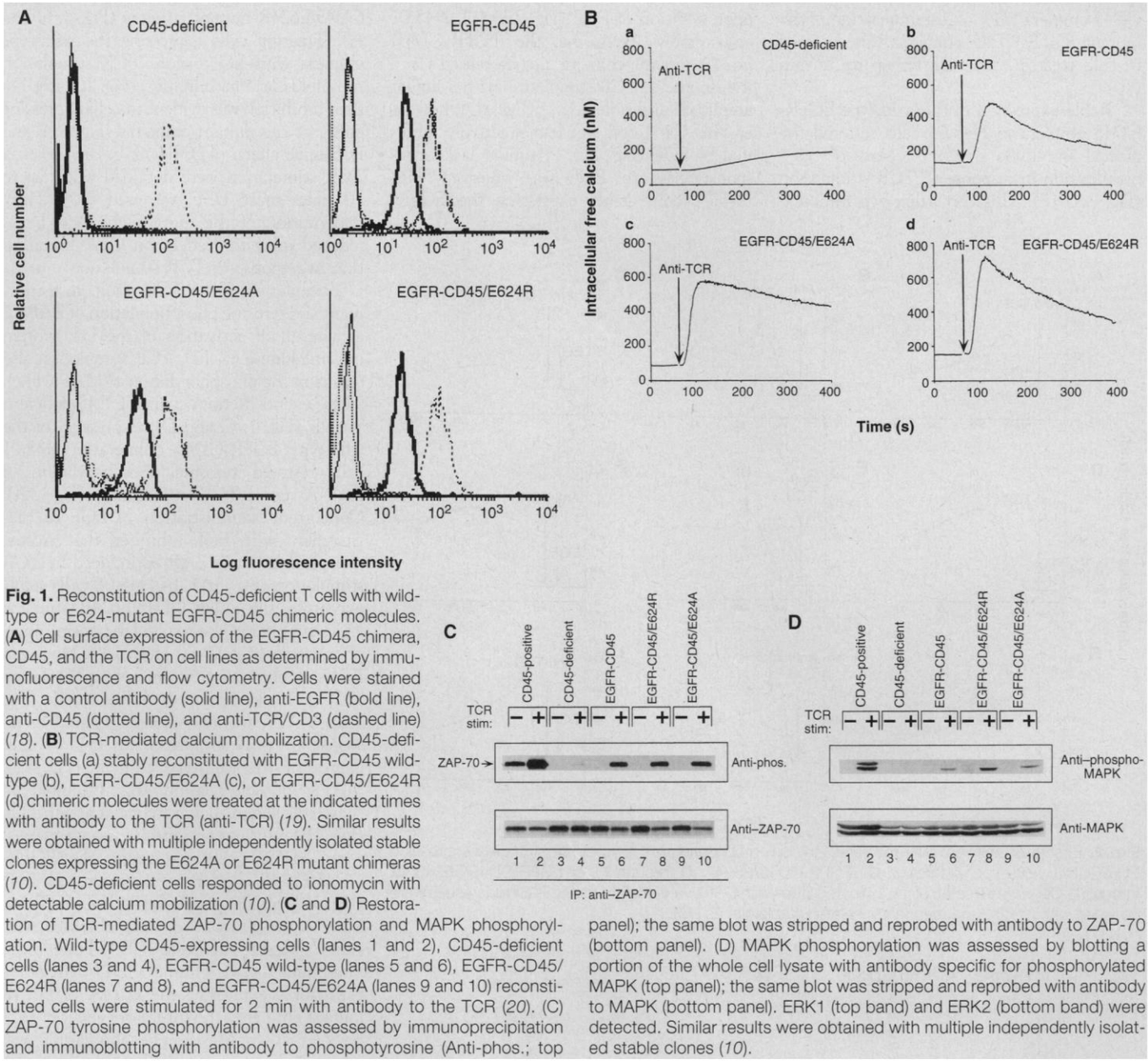

Fig. 1. Reconstitution of CD45-deficient T cells with wild-type or E624-mutant EGFR-CD45 chimeric molecules. (**A**) Cell surface expression of the EGFR-CD45 chimera, CD45, and the TCR on cell lines as determined by immunofluorescence and flow cytometry. Cells were stained with a control antibody (solid line), anti-EGFR (bold line), anti-CD45 (dotted line), and anti-TCR/CD3 (dashed line) (*18*). (**B**) TCR-mediated calcium mobilization. CD45-deficient cells (a) stably reconstituted with EGFR-CD45 wild-type (b), EGFR-CD45/E624A (c), or EGFR-CD45/E624R (d) chimeric molecules were treated at the indicated times with antibody to the TCR (anti-TCR) (*19*). Similar results were obtained with multiple independently isolated stable clones expressing the E624A or E624R mutant chimeras (*10*). CD45-deficient cells responded to ionomycin with detectable calcium mobilization (*10*). (**C** and **D**) Restoration of TCR-mediated ZAP-70 phosphorylation and MAPK phosphorylation. Wild-type CD45-expressing cells (lanes 1 and 2), CD45-deficient cells (lanes 3 and 4), EGFR-CD45 wild-type (lanes 5 and 6), EGFR-CD45/E624R (lanes 7 and 8), and EGFR-CD45/E624A (lanes 9 and 10) reconstituted cells were stimulated for 2 min with antibody to the TCR (*20*). (C) ZAP-70 tyrosine phosphorylation was assessed by immunoprecipitation and immunoblotting with antibody to phosphotyrosine (Anti-phos.; top panel); the same blot was stripped and reprobed with antibody to ZAP-70 (bottom panel). (D) MAPK phosphorylation was assessed by blotting a portion of the whole cell lysate with antibody specific for phosphorylated MAPK (top panel); the same blot was stripped and reprobed with antibody to MAPK (bottom panel). ERK1 (top band) and ERK2 (bottom band) were detected. Similar results were obtained with multiple independently isolated stable clones (*10*).

the catalytic site and the wedge containing the acidic residues.

To test this model, we stably reconstituted a CD45-deficient T cell line, H45.01 (*4*), with EGFR-CD45 chimeric molecules in which glutamate 624, analogous to aspartate 228 in the RPTPα wedge (*8*), was mutated to alanine (E624A) or arginine (E624R) (*9*). Subsequently, we assessed the ability of EGF to negatively regulate TCR signal transduction in these cells. Stable reconstitution of this CD45-deficient cell line with the wild-type EGFR-CD45 chimera restored normal TCR-mediated signal transduction (Fig. 1) (*7*). Wild-type and mutant reconstituted cell lines expressed comparable amounts of the EGFR-CD45 chimera and comparable amounts of the TCR/CD3 complex, as determined by flow cytometry (Fig. 1A). Mutation of glutamate 624 appeared not to result in a global defect in the function of CD45, because in H45.01 cells reconstituted with E624A or E624R mutant chimeras, mobilization of calcium (Ca^{2+}) (Fig. 1B) and increased phosphorylation of the protein tyrosine kinase ZAP-70 and mitogen-activated protein kinase (MAPK) (Fig. 1, C and D) in response to TCR stimulation were similar to those responses in cells reconstituted with the wild-type chimera. Tyrosine phosphatase activity of the mutant CD45 cytoplasmic domains expressed in *Escherichia coli* was similar to that of the wild-type protein (*10*). It is unlikely that these mutations affect EGF binding to the chimeric receptor, because the extracellular portion of all three chimeras consists entirely of the wild-type EGFR extracellular domain, and these cell lines displayed similar specific binding of radioiodinated EGF (*10*). As observed with the wild-type EGFR-

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CD45 chimera (*11*), no internalization of the mutant EGFR-CD45 chimeras was detected in cells treated with EGF for up to 30 min (*10*).

Stable expression of the wild-type EGFR-CD45 chimera in H45.01 cells restored the normal amplitude and time course of Ca^{2+} mobilization in response to TCR stimulation (Fig. 2A) (*7*). This Ca^{2+} flux was inhibited upon addition of EGF (Fig. 2B) (*7*). H45.01 cells stably expressing the EGFR-CD45/E624A mutant chimera also mobilized Ca^{2+} in response to TCR stimulation with a similar amplitude and time course (Fig. 2D); however, this Ca^{2+} flux was less effectively inhibited by EGF (Fig. 2E). A similar lack of responsiveness to EGF was observed with H45.01 cells stably expressing the EGFR-CD45/E624R mutant chimera (Fig. 2, G and H). Treating cells expressing the wild-type chimera with EGF before TCR stimulation inhibited Ca^{2+} mobilization (Fig. 2C) (*7*). No such inhibition was evident in cells expressing either of the mutant chimeras (Fig. 2, F and I). Similar effects of EGF both before and after TCR stimulation were obtained when up to 10 times more EGF was used (*10*). Thus, dimerization of the mutant chimeras has a reduced inhibitory effect on Ca^{2+} mobilization in response to TCR stimulation.

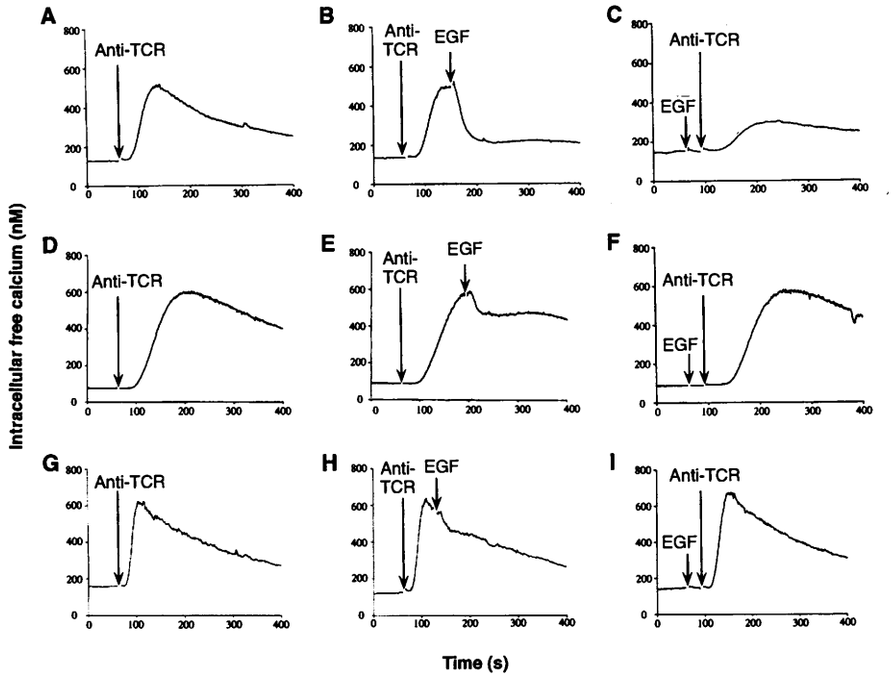

Fig. 2. Effects of EGF on TCR-mediated calcium mobilization in CD45-deficient T cells expressing EGFR-CD45 wild-type (**A** through **C**), EGFR-CD45/E624A (**D** through **F**), or EGFR-CD45/E624R (**G** through **I**). Cells were treated at the indicated times with EGF or antibody to the TCR. Similar results were obtained with multiple independently isolated stable clones (*10*).

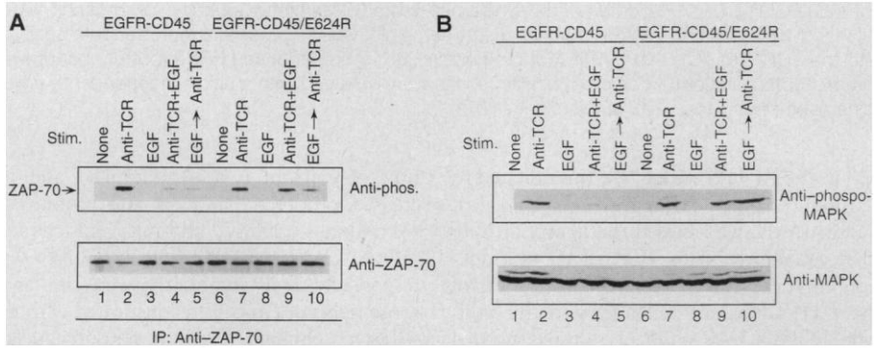

Fig. 3. Effects of EGF on TCR-mediated ZAP-70 and MAPK phosphorylation in CD45-deficient T cells expressing EGFR-CD45 wild-type (A and B, lanes 1 through 5) or EGFR-CD45/E624R (A and B, lanes 6 through 10). Cells were stimulated as indicated: no stimulation (lanes 1 and 6); 2 min with antibody to the TCR (lanes 2 and 7); 3 min with EGF (lanes 3 and 8); 2 min with both antibody to the TCR and EGF (lanes 4 and 9); and 1 min pretreatment with EGF, then 2 min with antibody to the TCR (lanes 5 and 10). (**A**) ZAP-70 tyrosine phosphorylation was assessed by immunoprecipitation, followed by immunoblotting with antibody to phosphotyrosine (top panel); the same blot was stripped and reprobed with antibody to ZAP-70 (bottom panel). (**B**) MAPK phosphorylation was assessed by blotting a portion of the whole cell lysate with antibody specific for phosphorylated MAPK (top panel); the same blot was stripped and reprobed with an antibody to MAPK (bottom panel). In the bottom panel, the variable upper band represents ERK1. Similar results were obtained with multiple independently isolated stable clones (*10*).

Stimulation of the TCR results in recruitment and tyrosine phosphorylation of ZAP-70 as a result of activation of the Lck protein tyrosine kinase (*5*, *12*). TCR stimulation also results in the phosphorylation of MAPK (*12*). These events do not occur in CD45-deficient T cells (Fig. 1, C and D). Expression of the wild-type EGFR-CD45 chimera in H45.01 cells restored tyrosine phosphorylation of ZAP-70 upon TCR stimulation (Fig. 3A). Concurrent administration of EGF or pretreatment with EGF inhibited the tyrosine phosphorylation of ZAP-70 induced by TCR stimulation (Fig. 3A). In H45.01 cells stably expressing the EGFR-CD45/E624R chimera, TCR stimulation also resulted in tyrosine phosphorylation of ZAP-70 (Fig. 3A). However, EGF's inhibition of tyrosine phosphorylation of ZAP-70 was reduced in these cells (Fig. 3A). MAPK was also phosphorylated in response to TCR stimulation in H45.01 cells expressing the wild-type chimera (Fig. 3B). MAPK phosphorylation was inhibited in these cells when they were treated with EGF (Fig. 3B). Little or no inhibition was observed when H45.01 cells expressing the EGFR-CD45/E624R chimera were treated with EGF (Fig. 3B). Similar results for both ZAP-70 and MAPK phosphorylation were obtained with cells expressing the EGFR-CD45/E624A mutant chimera (*10*). Thus, in CD45-deficient T cells stably expressing the E624A or E624R mutant chimeras, the inhibitory effect of EGF on ZAP-70 or MAPK phosphorylation was reduced or eliminated.

The hallmarks of T cell activation after TCR stimulation are production of interleukin-2 and increased cell proliferation, which require both activation of the Ras pathway (leading to MAPK phosphorylation) and Ca^{2+} mobilization (*12*). Here we have shown that both signaling pathways are less effectively inhibited by ligand induced dimerization of E624-mutant EGFR-CD45 chimeric molecules. Thus, it appears that T cells expressing E624A or E624R mutant CD45 molecules would continue to be activated in the presence of an inhibitory ligand.

Although ligands for several RPTPs have been determined (*1*), the natural ligand for CD45 remains unknown. CD45 can form dimers (*13*), and some antibodies that can dimerize CD45 inhibit its function (*14*). The

interaction of CD45 with its ligand may induce its dimerization and in turn regulate the activity of Lck. In the absence of ligand, both wild-type and mutant CD45 molecules are catalytically active monomers. In the presence of a CD45 ligand, both wild-type and mutant CD45 may dimerize, with different consequences for Lck activity. In cells expressing wild-type CD45, the catalytic site of each molecule would be blocked by the wedge containing glutamate 624 from the partner molecule, inhibiting CD45 phosphatase activity. Consequently, Lck would remain in the phosphorylated, inactive conformation, and TCR signals would be inhibited. In E624R-mutant CD45 molecules, the wedge is altered so that the catalytic sites are not occluded in the ligand-induced dimer. CD45 phosphatase activity would be retained and maintain Lck in its active conformation.

We chose to mutate glutamate 624 of CD45 because it is analogous to aspartate 228 within the putative inhibitory wedge of RPTPα (*8*). Aspartate 228 of one monomer contacts the mobile loop in the active site of the opposing monomer through a hydrogen bond between the side chain carboxyl moiety of aspartate 228 and a backbone amide of the loop. This interaction, along with other contacts, would preclude the necessary movement of the loop upon substrate binding, rendering the phosphatase inactive. Mutation of glutamate 624 of CD45 presumably disrupts the analogous interaction in CD45 dimers, thereby allowing the mobile loop to change conformation upon substrate binding, resulting in an active CD45 phosphatase.

Ligand-induced dimerization plays an essential role in the regulation of receptor tyrosine kinases, leading to autophosphorylation and activation of protein tyrosine kinase activity (*15*). Ligand-induced dimerization may also play an essential role in the regulation of RPTPs. However, instead of leading to activation, dimerization of RPTPs results in inhibition.

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9. The EGFR-CD45 chimera (consisting of residues 1 through 646 of the human EGFR and residues 580 through 1281 of human CD45, numbered after the signal sequence from isoform ABC) has been described (*7*). The E624A and E624R mutations were introduced into the wild-type EGFR-CD45 chimera by oligonucleotide-based mutagenesis using the polymerase chain reaction and confirmed by nucleotide sequencing. H45.01 (*4*) cells (a CD45-deficient derivative of the HPB.ALL T cell line) were transfected with plasmids encoding the EGFR-CD45 mutant chimeric molecules. Subsequent limiting dilution and selection in geneticin-containing medium (2 mg/ml) yielded H45XLE624A.5 (expressing the EGFR-CD45/E624A mutant chimera) and H45XLE624R.3 (expressing the EGFR-CD45/E624R mutant chimera). H45XL2 (expressing the EGFR-CD45 wild-type chimera) was derived in a similar manner (*7*). Multiple clones expressing each of the chimeric molecules were isolated and analyzed.
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18. Cells were stained with a control monoclonal antibody (mAb) [goat antibody to mouse immunoglobulin G (Caltag Laboratories)]; with LA22 (antibody to EGFR; Upstate Biotechnology, Lake Placid, NY) for the EGFR-CD45 chimera; with Hle-1 (Becton-Dickinson) for CD45; and with Leu4 (antibody to CD3ε; Becton-Dickinson) for the TCR. Cells were stained at 4°C with saturating concentrations of fluorescein isothiocyanate (FITC)–conjugated primary antibody (control, Hle-1, and Leu4) or primary antibody (LA22) followed by FITC-conjugated goat antibody to mouse IgG. Cells were analyzed on a FACScan (Becton-Dickinson).
19. The intracellular free Ca^{2+} concentration was measured with the calcium-sensitive dye Indo-1 as described (*16*). Cells (5×10^6 per milliliter) were treated with antibody to CD3 (mAb 235 at a 1:3000 dilution of ascites) or EGF (100 ng/ml).
20. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended at 2×10^8 per milliliter in PBS. Cells were incubated at 37°C for 15 min. For each sample, 2×10^7 cells were used, and an equal volume of stimulus in PBS warmed to 37°C was added for the indicated time so that final conditions were as follows: cells, 1×10^8 per milliliter; 235 ascites (mAb to CD3) at 1:500 dilution; and EGF, 100 ng/ml. Cells were sedimented in a microfuge and lysed in 200 μl of lysis buffer [1% Triton X-100, 150 mM NaCl, and 10 mM tris (pH 8.0), supplemented with protease and phosphatase inhibitors as described (*11*)]. Lysates were incubated at 4°C for 30 min, followed by centrifugation at 13,000*g* for 10 min. Ninety percent of the lysate was subjected to immunoprecipitation with polyclonal rabbit antibody to ZAP-70 [1598 (*17*)] and protein A–Sepharose beads for 2 hours at 4°C, after which immune complexes were washed. Immune complexes and 10% of the untreated lysate were resolved separately by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). Immunoblotting was done with mAb to phosphotyrosine (4G10; Upstate Biotechnology) or anti-phospho-MAPK (New England Biolabs), followed by visualization by enhanced chemiluminescence (Amersham). The blots were stripped and reprobed with antibody to ZAP-70 (1598) or antibody to MAPK (Santa Cruz Biotechnology), respectively.

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Dissociated Pattern of Activity in Visual Cortices and Their Projections During Human Rapid Eye Movement Sleep

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Positron emission tomography was used to measure cerebral activity and to evaluate regional interrelationships within visual cortices and their projections during rapid eye movement (REM) sleep in human subjects. REM sleep was associated with selective activation of extrastriate visual cortices, particularly within the ventral processing stream, and an unexpected attenuation of activity in the primary visual cortex; increases in regional cerebral blood flow in extrastriate areas were significantly correlated with decreases in the striate cortex. Extrastriate activity was also associated with concomitant activation of limbic and paralimbic regions, but with a marked reduction of activity in frontal association areas including lateral orbital and dorsolateral prefrontal cortices. This pattern suggests a model for brain mechanisms subserving REM sleep where visual association cortices and their paralimbic projections may operate as a closed system dissociated from the regions at either end of the visual hierarchy that mediate interactions with the external world.

Since its discovery in 1953 (*1*), the stage of sleep characterized by electroencephalographic desynchronization and rapid eye movements (REM sleep) has been the subject of unremitting scientific investigation. Exceptional interest in REM sleep