duced in $CD28^{-/-}$ mice. Whereas the amount of IgM is normal, the pattern of IgG subclasses in CD28-deficient mice is altered. The nature of the changes in T cell–dependent IgG subclasses in $CD28^{-/-}$ mice could lie in an altered pattern of cytokines that are involved in Ig isotype regulation (*39*). Disruption of CD28-B7 interaction with soluble CTLA4Ig suppresses T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin (*22*). To investigate whether these results are due to impaired T or B cell function, we infected CD28 mutant mice with VSV. Our data show that B cell responsiveness is unaltered in the absence of CD28, as indicated by the normal titers of neutralizing anti-VSV IgM, which are independent of T cell helper activity (*34*). However, the class switch to IgG, which is dependent on the function of T helper cells (*34*), was significantly reduced, suggesting that T help is diminished in the absence of costimulation by CD28.

In conclusion, our data indicate that CD28 costimulation is differentially required for cell-mediated and humoral immune responses in vivo. The CD28-deficient mice will be a valuable tool to further elucidate the role of costimulatory events by CD28-dependent and -independent mechanisms in the generation of immune responses against pathogens and tumors as well as in the course of autoimmune diseases. The study of these animals could help determine where immunosuppression by disruption of CD28-B7 interaction can be effective as a treatment strategy as well as when T cell activation is dependent on other mechanisms of costimulation.

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41. We thank N. Ramachandra for technical assistance, D. R. Koh for support, and V. A. Wallace, J. Penninger, and P. Waterhouse for critical reading of the manuscript. Supported by an AIDS-Stipendienprogramm fellowship of the German government (to K.P.), Swiss National Science Foundation fellowship (to T.M.K.), Medical Research Council fellowship of Canada (to K.Ki.), Medical Research Council of Canada, the National Science and Engineering Research Council of Canada, and the National Cancer Institute of Canada.

17 May 1993; accepted 21 June 1993

Interaction of Activated EGF Receptors with Coated Pit Adaptins

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The epidermal growth factor (EGF) receptor interacts with plasma membrane–associated adapter proteins during endocytosis through coated pits. Almost 50 percent of the total pool of α-adaptins was coimmunoprecipitated with the EGF receptor when A-431 cells were treated with EGF at 37°C, but not at 4°C. Partial proteolysis of α-adaptin suggested that the amino-terminal domain is the region that associates with the EGF receptor. The extent of receptor-adaptin association was increased in cells depleted of potassium to block endocytosis. These data suggest that receptor-adaptin association occurs in intact cells before coated pits are fully assembled.

A large variety of extracellular molecules (for example, nutrient carriers, growth factors, and peptide hormones) and viruses interact with the surface of mammalian cells and rapidly enter the cell through specialized coated pit regions of the plasma membrane (*1*, *2*). Clathrin-coated pits and vesicles have been implicated in the selective recruitment of membrane proteins in receptor-mediated endocytosis (*1*, *2*). However, the mechanism of this dynamic process, which must involve interactions between the cytoplasmic tails of receptors and coated pit proteins, is poorly understood, particularly for ligand-induced endocytosis. For example, binding of EGF to its receptor on the cell surface accelerates receptor endocytosis and results in a rapid loss of receptors (*3*–*6*). This ligand-induced downregulation is a general phenomenon for all receptor tyrosine kinases (*3*–*7*) and an important part of the signal transduction events initiated by the growth factors (*8*). Growth factor binding allows receptors, which are diffusely distributed at the cell surface, to cluster in clathrin-coated pits and, thereafter, to be rapidly internalized (*5*, *9*).

The main components of plasma membrane coated pits are AP-2, assembly or adapter proteins, and clathrin (*1*, *2*, *10*).

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AP-2 is a heterotetrameric complex [two proteins of ~100 kD, termed α- and β-adaptins, and one 50-kD and one 16-kD protein, (*10, 11*)], that anchors the clathrin lattice to the inner surface of the plasma membrane (*12*). It may serve as a binding site for the intracellular domains of nutritive receptors (*13*). However, it is not known whether AP-2 directly associates with receptors in vivo or, if so, at what stage of endocytosis this occurs.

The current model of the coated pit cycle assumes that interaction of the receptor with coated pit proteins should be transient (*1, 2*). To test whether receptors associate with adapter proteins during EGF-induced endocytosis, we used A-431 cells, which express a large amount of EGF receptors. In these cells the receptors will synchronously accumulate in coated pits after the addition of EGF at 37°C, but not at 4°C (*9*). Surface receptors were first saturated with EGF by incubating cells with EGF at 4°C for 1 hour, then the temperature was shifted to 37°C for 12 min to permit endocytosis (Fig. 1). Cell membranes were then solubilized in the buffer TGH (*14*), which contained Triton X-100, and the EGF receptor was immunoprecipitated (*14*). We tested for the presence of AP-2 in the immunoprecipitates by blotting with an antibody specific to α-adaptin, AC1-M11 (*15*). A very small amount of adaptins (similar to that in preimmune serum precipitates) was present in receptor immunoprecipitates obtained from the cells incubated with EGF at 4°C. Incubation at 37°C, however, increased the amount of coprecipitated α-adaptins (Fig. 1). This specific receptor-adaptin association was detected within 2 to 3 min of the start of the 37°C incubation, reached a maximum at 10 to 14 min, and then gradually declined. Similar results were obtained when another monoclonal antibody to α-adaptin, AC2-M15 (*15*), was used.

Because proteins composing AP-2 dissociate only under strong denaturing conditions (*2, 10, 11*), we expected to find β-adaptins in the EGF receptor immunoprecipitates. Indeed, β-adaptin was detected with a specific antibody (*15*) in immunoprecipitates from cells exposed to EGF at 37°C (*16*). Although most cellular clathrin was solubilized in TGH, as detected by protein immunoblotting with antibody to the clathrin heavy chain, we did not find clathrin in the EGF receptor immunoprecipitates. Therefore, adaptins seem to interact more avidly with receptors than with clathrin. Several specific but unidentified proteins were found in EGF immunoprecipitates obtained from metabolically ^{35}S-labeled cells that had been treated with EGF at 4°C (*16*). The amount of these proteins in receptor immunoprecipitates decreased when EGF-treated cells were further incubated at 37°C. In contrast, two proteins (~103 kD and 106 kD) were detected in the immunoprecipitates from cells exposed to EGF at 37°C, but not at 4°C. Immunoblotting of the EGF receptor immunoprecipitates indicated that these bands corresponded to β- and α-adaptins, respectively.

Thus, EGF receptor immunoprecipitation experiments revealed a temperature-dependent specific association of the EGF-activated receptor with AP-2. This result was reproduced by immunoprecipitation of receptors with antibody to phosphotyrosine. When EGF activates its receptor tyrosine kinase, this leads to tyrosine phosphorylation of cellular proteins and autophosphorylation of the receptor (*3, 7*). Both the phosphorylation of substrate proteins and receptor autophosphorylation occur at 4°C and typically are less dramatic at 37°C (*14, 17*). However, α-adaptins were detected in the phosphotyrosine immunoprecipitates when cells were incubated at 37°C, but not at 4°C (Fig. 1). This indicates that receptor autophosphorylation is not sufficient for adaptin coprecipitation, although it can be important for the receptor-adaptin interaction at 37°C. Furthermore, we detected a specific association of the phosphorylated EGF receptor with adaptins in cells exposed to low concentrations of EGF (1 to 5 ng/ml), conditions that activate a limited pool (1 to 10%) of EGF receptors (Fig. 1). Protein immunoblotting with several types of phosphotyrosine antibodies did not reveal any tyrosine phosphorylation of adaptins at 4° or 37°C. Because the EGF receptor was the major tyrosine-phosphorylated protein and contained more than 90% of the total cellular phosphotyrosine recovered from the EGF-treated A-431 cells, adaptins were probably coprecipitated with the phosphorylated EGF receptor. However, the participation of another tyrosine-phosphorylated protein cannot be ruled out.

We tested whether the association of EGF receptors with adaptins could be controlled in vivo, for example, by the rate of endocytosis, with a K^+-depletion technique (*18*). The depletion of K^+ blocks endocytosis by preventing the normal assembly of coated pits (*18*). It has been shown that adaptins remain associated with the plasma membrane in K^+-depleted HEp2 cells (*19*). Immunofluorescence staining of A-431 cells with α-adaptin antibody revealed that adaptins remained associated with the plasma membrane in K^+-depleted cells, although the intensity of staining was slightly weaker than in control cells. We found that K^+ depletion reduced endocytosis of ^{125}I-labeled EGF in A-431 cells by 85%. This block of coated pit endocytosis did not prevent receptor-adaptin interaction; rather, it resulted in an increased amount of α-adaptins in complexes immunoprecipitated with antibodies to the EGF receptor or to phosphotyrosine from both untreated and EGF-treated cells (Fig. 2). Under these conditions blocking endocytosis may reduce

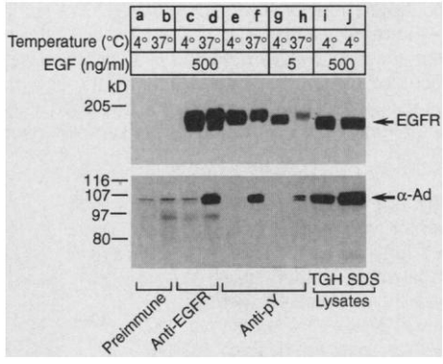

Fig. 1. Detection of α-adaptin complexes immunoprecipitated with antibodies to the EGF receptor and phosphotyrosine. We incubated A-431 cells with EGF (5 or 500 ng/ml) at 4°C for 1 hour and then incubated them in the same medium for an additional 12 min at 4° or 37°C. At the end of the second incubation, the cells were washed with Ca^{2+}, Mg^{2+}–free phosphate buffer and solubilized for 10 min at 4°C in TGH buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes, pH 7.3, 1 mM sodium orthovanadate, and protease inhibitors) (*14*). Proteins from cell lysates, representing equal amounts of cells, were immunoprecipitated with polyclonal antibody to the EGF receptor (anti-EGFR) (*2, 14*) (lanes c and d) or polyclonal antibody to phosphotyrosine (anti-pY) (Zymed) (lanes e through h) and protein A–Sepharose (Sigma). Preimmune rabbit serum was used as a control (lanes a and b). We washed immunoprecipitates twice with TGH and once with TGH containing 100 mM NaCl. Electrophoresis, transfer to nitrocellulose paper, and protein immunoblotting were done as described (*14, 17*). The top (above the 116-kD marker) and bottom portions of the nitrocellulose membrane were blotted with monoclonal anti-EGFR (UBI) and monoclonal antibody to α-adaptin, AC1-M11 (anti–α-Ad) (*15*), respectively. Goat antibodies to mouse immunoglobulin G labeled with ^{125}I or conjugated with horseradish peroxidase were used as secondary antibodies and detected with x-ray film or a Phosphorimager (Molecular Dynamics) or by enhanced chemiluminescence (Amersham). To determine the total amount of cellular adaptins relative to that recovered in immunoprecipitates, we solubilized parallel cultures in TGH (lane i) or SDS containing buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 50 mM tris-HCl, pH 8.5, and protease inhibitors) (lane j). Lysates equal to 65% of that used for immunoprecipitation were electrophoresed and blotted with anti–α-Ad or anti-EGFR. Typically, 30 to 40% of the total cellular pool of α-adaptins was soluble in TGH (compare lanes i and j). (EGFR, EGF receptor; α-Ad, α-adaptin.)

the dissociation of receptor-adaptin complexes that normally would dissociate after the formation of coated vesicles from coated pits (*1*). A reduction in dissociation that results from incubation of the cells in the absence of K^+ could produce the observed accumulation of receptor-adaptin complexes (Fig. 2). No differences in the extent of tyrosine phosphorylation or the amount of immunoprecipitated receptor were detected between control and K^+-depleted cells. Thus, the results from the K^+ depletion experiments suggest that the receptor association with adaptins can be modulated in vivo and that the receptor-adaptin complexes detected by coimmunoprecipitation are formed in vivo before cell solubilization.

There is other evidence of an in vivo association of the EGF receptor with adaptins. Quantitation of the data from coprecipitation assays (Figs. 1 and 2) revealed that, despite dilution of the interacting components during solubilization, as much as 30 to 50% of the total pool of α-adaptins present in the lysate was coprecipitated with activated EGF receptors. Moreover, the amount of α-adaptin extracted by TGH from cells exposed to EGF at 37°C was 1.5 to 2 times that extracted from cells not exposed to EGF at 37°C or from cells incubated with EGF at 4°C (Fig. 2). Therefore, AP-2 binding to activated EGF receptors seems to redistribute adaptins to a Triton X-100–soluble membrane fraction. Taken together, these data support the notion that the receptor-adaptin complexes exist in vivo and are retained after cell solubilization.

The results obtained with A-431 cells (Figs. 1 and 2) have been reproduced in other human epidermoid (squamous) carcinoma cell lines that express large amounts of EGF receptors (*20*). Specific association of the activated EGF receptor with adaptins has also been detected in normal human fibroblasts that express a moderate number of EGF receptors (*4*). Incubation at 37°C of fibroblasts that had been preincubated with EGF at 4°C substantially increased the amount of α-adaptin coprecipitated with the EGF receptor (Fig. 3). The depletion of K^+ also increased the extent of the receptor-adaptin association in these cells. A maximum of 5 to 10% of the total TGH-soluble pool of α-adaptin was associated with the phosphorylated receptor. Thus, the association with AP-2 is a general feature of EGF receptor endocytosis in normal and transformed cells.

The adaptin molecule consists of a COOH-terminal ear or appendage domain and an NH_2-terminal head domain connected by a hinge domain that contains sites of proteolytic cleavage (*21*). The digestion of AP-2 by elastase releases the appendage domains from the AP-2 core containing head domains of α- and β-adaptins and 50- and 16-kD proteins (*10*, *21*). On the basis of sequence data, researchers have speculated that the "ears" interact with receptors whereas NH_2-domains anchor AP-2 to the plasma membrane (*10*). To determine which domain of α-adaptin is involved in the interaction with the EGF receptor, cell lysates were incubated with elastase before immunoprecipitation with antibody to the EGF receptor. Alternatively, elastase was added to immunoprecipitates. Disappearance of the intact 100-kD adaptins from the immunoprecipitates of EGF-activated receptor correlated with the appearance of a 60-kD product of α-adaptin proteolysis that corresponds to the head domain (Fig. 4). These experiments suggest that the NH_2-terminal domain is required for adaptin association with the EGF receptor.

In conclusion, our results demonstrate an in vivo association of the EGF receptor with AP-2. Although unoccupied receptors do associate with the adaptins, binding of EGF to the receptor increased the extent of adaptin association. The interaction of adaptin with an activated receptor tyrosine kinase is unusual in that adaptin does not contain a Src homology 2

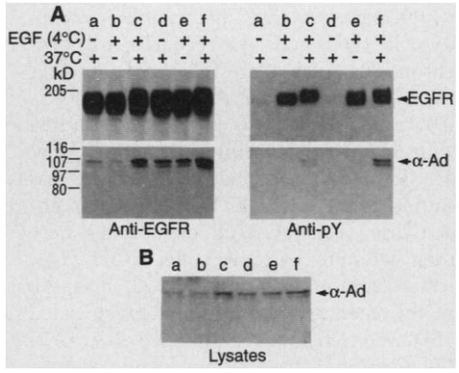

Fig. 2. Association of α-adaptins with the EGF receptors in K^+-depleted cells. A-431 cells were left untreated (lanes a through c) or hypotonically shocked for 5 min and incubated in K^+-free medium (lanes d through f) for 1 hour at 37°C (*18*). Untreated and K^+-depleted cells were incubated without (−) or with (+) EGF (500 ng/ml) at 4°C for 1 hour and then incubated at 37°C (+) or left at 4°C (−) for 15 min in regular (lanes a through c) or K^+-free (lanes d through f) medium. (**A**) Two portions each representing 45% of the total TGH-solubilized lysate were used for precipitation with anti-EGFR (left panel) or anti-pY (right panel), and (**B**) 10% of the lysate was directly processed by electrophoresis and blotting. We detected the receptor (EGFR) and α-adaptins (α-Ad) in the immunoprecipitates and lysates as described (Fig. 1).

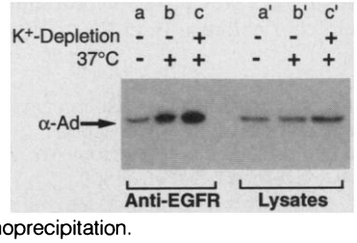

Fig. 3. Association of EGF receptor with adaptin in human fibroblasts. Cells were left untreated (−) or subjected to the depletion of K^+ (+) as described (Fig. 2). Cells were then incubated with EGF (100 ng/ml) for 1 hour at 4°C and incubated additionally for 15 min at 37°C (+) or left at 4°C (−) in regular (lanes a, b, a′, and b′) or K^+-free medium (lanes c and c′). We detected α-adaptins (α-Ad) in EGF receptor immunoprecipitates (lanes a through c) and cell lysates (lanes a′ through c′) as described (Fig. 1). Lysates represent 5% aliquots of the samples used for immunoprecipitation.

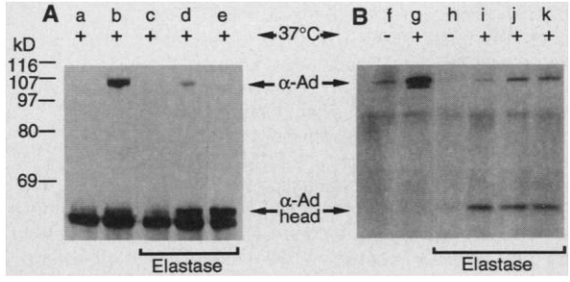

Fig. 4. Interaction of the EGF receptor with the NH_2-domain of α-adaptin. We incubated A-431 cells with EGF (500 ng/ml) at 4°C and then incubated them for 12 min at 37°C (+) or left them at 4°C (−) as described (Fig. 1). (**A**) Cells were solubilized in TGH buffer without protease inhibitors, and lysates (100 μg of protein per sample) were left untreated (lanes a and b) or incubated with 125 ng (lanes c and d) or 625 ng (lane e) of elastase for 15 min at 4°C. Phenylmethylsulfonyl fluoride (2 mM) was used to inactivate elastase, and the EGF receptors were then immunoprecipitated. Lysates in lanes a and c were incubated with preimmune serum instead of EGF receptor antiserum. (**B**) We immunoprecipitated EGF receptors as described (Fig. 1). The immunoprecipitates were left untreated (lanes f and g) or incubated with elastase (125 ng per immunoprecipitate obtained from 4×10^5 cells) (lanes h through k) for 5 (lane k), 10 (lane j), or 15 min (lanes h and i) at 4°C and washed with TGH containing protease inhibitors. Electrophoresis of immunoprecipitates and protein immunoblotting of intact α-adaptins (α-Ad) and the NH_2-terminal degradation product of α-adaptin (α-Ad head) were performed as described (Fig. 1). The band of ~55 kD recovered from both the preimmune and immune serum (A) was detected by the secondary antibodies alone, if not digested by elastase.

(SH2) domain (*22*). Unlike receptor-SH2 interactions, EGF receptor–adaptin association does not occur at 4°C. Moreover, the extent of receptor-adaptin association is greater than that between receptors and proteins that contain SH2 domains (*22*). Alternatively, the interaction between the EGF receptor and adaptins may be more stable than the association of the growth factor receptors with SH2 domain–containing proteins.

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23. We thank M. S. Robinson (University of Cambridge, Cambridge, United Kingdom) for the antibodies to adaptins and helpful discussions, F. Brodsky for the antibody to the clathrin heavy chain (X-22), and T. Sorkina and U. Barnela for technical assistance. Supported by National Cancer Institute grant CA24071.

15 March 1993; accepted 27 May 1993

Functional Magnetic Resonance Imaging of Motor Cortex: Hemispheric Asymmetry and Handedness

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A hemispheric asymmetry in the functional activation of the human motor cortex during contralateral (C) and ipsilateral (I) finger movements, especially in right-handed subjects, was documented with nuclear magnetic resonance imaging at high field strength (4 tesla). Whereas the right motor cortex was activated mostly during contralateral finger movements in both right-handed (C/I mean area of activation = 36.8) and left-handed (C/I = 29.9) subjects, the left motor cortex was activated substantially during ipsilateral movements in left-handed subjects (C/I = 5.4) and even more so in right-handed subjects (C/I = 1.3).

Two basic aspects of human motor function are hemispheric dominance and handedness. The dominant roles of the left hemisphere for speech and the right hemisphere for visuospatial tasks and for the spatial distribution of attention are well documented (*1*). With regard to motor function, the deficits resulting from a left hemispheric lesion are more pronounced than those resulting from a right hemispheric lesion (*2*), although some specific deficits depend on the task (*3*). Damage to the cortex around the central sulcus, or lesion of its output at the internal capsule, leads to paralysis or paresis of the contralateral hand, but the motor function of the ipsilateral hand is differentially affected by lesions in the left or the right hemisphere: Left hemispheric lesions result in motor dysfunction of the ipsilateral (left) hand, whereas lesions on the right side leave the motor function of the ipsilateral (right) hand relatively unaffected (*2*). However, little attention has been paid to the handedness of subjects whose motor functions have been studied after cortical brain damage (*2*, *3*). We assessed the possible asymmetry of activation in the right and left motor cortex and tried to determine the relations, if any, between this activation and handedness.

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Fifteen healthy human subjects (*4*) participated in the study; ten were right-handed (five women and five men) and five were left-handed (one woman and four men) (*5*). Subjects lay supine in a 4-T nuclear magnetic resonance (NMR) instrument (*6*). They were instructed to make repetitive opposition movements of the thumb on each of the remaining four fingers and to exert moderate pressure at each contact while NMR images of each hemisphere were acquired before, during, and after the task (Fig. 1) (*7*). For each hemisphere, the task was performed successively by each hand in random order. The surface area of activation within the precentral gyrus of each hemisphere during the movement of each hand was calculated (*8*), and differences in this area between the activation obtained during movements of the contralateral and ipsilateral fingers were determined (*9*). The ratio of the mean area of contralateral/ipsilateral (C/I) activation was also calculated. This study extends previous work on functional activation of the motor cortex that was done with high-field NMR (*10*).

There was a hemispheric asymmetry in the functional activation of the motor cortex during contralateral and ipsilateral movements, especially in right-handed subjects (Figs. 2 and 3) (*11*). Thus, whereas the right motor cortex was activated mostly during contralateral finger movements in both right-handed (C/I = 36.8) and left-handed (C/I = 29.9) subjects, the left motor cortex was activated substantially during ipsilateral movements in left-handed subjects (C/I = 5.4) and even more so in right-handed subjects (C/I = 1.3).

The involvement of the left motor cortex in ipsilateral hand movements could explain the well-documented significant deficits produced by left, but not right, hemispheric lesions on the motor performance of the ipsilateral hand (*2*, *3*). In general, some activation of the human mo-