# Role of the regulatory domain of the EGF-receptor cytoplasmic tail in selective binding of the clathrin-associated complex AP-2

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**Background:** After stimulation of a cell by the mitogenic epidermal growth factor (EGF), the EGF receptor (EGF-R) is cleared from the cell surface in order to turn off receptor signaling. This internalization is mediated via clathrin-coated pits and coated vesicles, and ultimately the receptors are delivered to the lysosome and destroyed. It is believed that clathrin-associated protein complexes or adaptors (APs) link the entrapment of EGF-R and other nutrient and growth-factor receptors to the formation of the clathrin-coated pit. Two classes of APs are known — AP-2, found at the plasma membrane, and AP-1, found in the trans-Golgi network. Activated EGF-R associates with AP-2s at the plasma membrane, but the mechanism responsible for this association is not known. Here, we investigate, *in vivo* and *in vitro*, three aspects of the interaction between APs and EGF-R: firstly, we ask whether EGF-R at the plasma membrane distinguishes between AP-1 and AP-2; secondly, we ask which part of the receptor's cytoplasmic tail is responsible for binding; finally, we ask whether autophosphorylation by EGF-R is essential for the interaction.

**Results:** We demonstrate that EGF-R displays a selective association for AP-2 over AP-1 *in vivo*, and that this preferential interaction can also be detected using surface plasmon resonance *in vitro*. Using a truncated mutant and a kinase-dead mutant of EGF-R, we show that the regulatory domain of the cytoplasmic tail is essential for the recruitment of AP-2 *in vivo* and that this domain is required for association between purified AP-2 and EGF-R *in vitro*. Finally, we demonstrate, *in vivo* and *in vitro*, that tyrosine auto-phosphorylation by the receptor is not an essential pre-condition for the recruitment of AP-2.

**Conclusions:** EGF-R binds selectively to AP-2s, and the regulatory domain of its cytoplasmic tail is required for this interaction. The lack of correlation between receptor autophosphorylation and AP-2 recruitment suggests that activation of the EGF-R kinase stimulates endocytosis by the phosphorylation of a factor distinct from EGF-R itself, as also proposed by others based on experiments measuring receptor traffic and entrapment.

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## Introduction

Two classes of receptors are known to be efficiently internalized by the clathrin-coated pit/coated-vesicle pathway (reviewed in [1]). One class includes nutrient receptors such as the low-density lipoprotein receptor, the asialoglycoprotein receptor and the transferrin receptor — proteins that internalize rapidly from the cell surface whether their ligands are present or not. The second class includes growth-factor receptors such as the epidermal growth-factor receptor (EGF-R) and the platelet-derived growth-factor receptor, which must associate with their ligands in order to be recruited by coated pits. In many instances, this ligand-induced endocytosis removes the activated receptor from the cell surface, resulting in its down-regulation.

The clathrin-associated protein complexes (APs), also referred to as 'adaptors' or 'assembly proteins', are two related but distinct heterotetramers: AP-1, specific to the trans-Golgi network (TGN), and AP-2, specific to the plasma membrane (see [2,3]). AP-1 contains the large chains $\gamma$ and $\beta1$, the medium chain $\mu1$ and the small chain $\sigma1$; AP-2 contains the large chains $\alpha a$ or $\alpha c$ and $\beta2$, the medium chain $\mu2$ and the small chain $\sigma2$. APs promote the assembly of the clathrin lattice [4,5], and are located between the lattice and the cytoplasmic surface of the membrane in coated pits and coated vesicles [6,7]. Their location, together with biochemical data [8–11] showing an association between purified APs and isolated cytoplasmic tails of receptors that undergo constitutive traffic, has led to a model in which the recruitment of both classes of receptors into clathrin-coated pits is mediated by interactions of APs with receptor tails.

The detailed mechanism underlying this traffic through coated pits is not known, but several ideas have emerged from *in vitro* binding studies between receptors and AP complexes, and from kinetic studies of endocytosis in living cells, to explain the specificity of this process. Entrapment of a receptor onto a coated pit might occur because an AP complex, already localized on the plasma membrane or on the TGN, recognizes and associates with a sorting signal on the receptor. These signals, found in the cytoplasmic tails of nutrient and growth-factor receptors and mostly identified by mutational analysis, typically consist of four to six amino acids, which do not display strict sequence conservation (see [1]). The receptor–AP

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interaction would then increase the affinity of AP complexes for cytosolic clathrin, resulting in the rapid assembly of the coat and the eventual capture of the receptor in a coated vesicle. Alternatively, a receptor that freely diffuses in the plane of the membrane could interact with APs already forming coated pits and about to form coated vesicles.

The recent demonstration that ligand-activated EGF-Rs located at the cell surface associate with plasma membrane AP-2 complexes — the first example of receptor–AP association in living cells — has enhanced our understanding of receptor recruitment into clathrin-coated structures [12,13]. The cytoplasmic tail of EGF-R contains a membrane-proximal tyrosine kinase and a membrane-distal regulatory domain of about 230 amino acids [14]. The regulatory domain has a 48 amino-acid region, which contains at least three sequences that provide endocytic signals, and a so-called 'inhibitory domain' of about 160 amino acids at the carboxyl terminus of the molecule, which contains the autophosphorylation sites [14,15]. It is believed that the binding of EGF to the extracellular part of the receptor leads to receptor dimerization and stimulation of the tyrosine kinase domain, allowing *trans*-autophosphorylation of the receptor tails [16]. Several tyrosine residues in the inhibitory domain are phosphorylated after activation [17–19]. These modifications cause the recruitment of several proteins, notably Grb-2 and Sos [20], which initiate signal transduction via the Ras pathway. At the same time, the EGF-Rs, which are normally free to diffuse in the plane of the membrane, become clustered in coated pits and are rapidly internalized (see [21]).

It has been proposed that phosphorylation of the inhibitory domain induces a conformational change, which allows the endocytic signals within the 48 amino-acid sub-domain of the regulatory region to interact with the endocytic apparatus [22]. However, dimerization and autophosphorylation of the receptor do not appear to be sufficient to activate EGF-R internalization. For example, 'kinase-dead' EGF-R mutants — with an inactive kinase but an otherwise intact receptor tail — will not endocytose in response to EGF, although presumably they can still dimerize [23,24]. Likewise, certain EGF-R deletion mutants, lacking the autophosphorylation sites but containing the endocytic signals, are actively internalized in response to EGF, but only when the receptor bears an active kinase [15]. Together with studies on the internalization of other receptors [25–27], these results suggest that the kinase activity is required to stimulate receptor endocytosis by the phosphorylation of a target distinct from the receptor itself.

Here, we show that the EGF-R regulatory domain containing the endocytic signals is essential for the recruitment of AP-2 complexes by activated receptors in living cells. Moreover, we reproduce this association *in vitro*, using isolated EGF-R and purified AP-2 complexes. We find that tyrosine autophosphorylation of EGF-R is not an essential pre-condition for AP-2 recruitment, as a kinase-dead mutant of EGF-R can also associate with AP-2 complexes both *in vivo* and *in vitro*. Together with the results summarized above, our data are consistent with the model in which the most important event required for EGF-R–AP association is the phosphorylation of a substrate distinct from either the receptor or the APs. How this second event is linked to AP-recruitment remains to be determined.

## Results

### The regulatory domain of the EGF-R is required for the interaction of the receptor with AP-2 complexes *in vivo*

We used two procedures to confirm that human EGF-R, expressed in mouse B82L cells transfected with the human *EGF-R* gene, associates with AP-2. In the first, as described by Sorkin and Carpenter [12], cells were incubated at 4 °C with human EGF to activate the kinase and to induce receptor autophosphorylation; coated-pit formation and internalization do not occur at this temperature. The activated cells were then warmed to 37 °C to re-establish endocytosis and to allow association of EGF-R with AP-2 complexes. Immunoprecipitation of EGF-R, together with western blotting, indicated that EGF-R indeed associated with AP-2 complexes and autophosphorylated (Fig. 1, lanes 1–7). The largest increase in the strength of the signal (about eight-fold), corresponding to association with the AP-2 complexes of the plasma membrane, was usually obtained after 20–60 minutes of rewarming; this increase also corresponded to association with 1–2 % of the total cellular pool of AP-2 — in agreement with results obtained recently using mouse NIH3T3 cells that express human EGF-R [13].

In the second procedure, the cells were kept at 37 °C during the incubation with EGF. The results were similar to those of the first procedure, although a faster rate of EGF-R–AP-2 association was observed (Fig. 1, lanes 15–21) — more consistent with the known course of EGF-R internalization in transfected B82L cells [28]. By contrast, this temperature effect was not seen in Chinese hamster ovary (CHO) cells transfected with the human *EGF-R* gene, where rapid association and dissociation phases were observed even with pre-cooling (see Fig. 8, below). The association of AP-2 complexes with EGF-R was specific, as no bands reacting with anti-α antibody were detected in control experiments performed with untransfected mouse B82L cells (Fig. 1, lanes 8–14).

A truncated mutant of EGF-R (c'958), lacking the regulatory domain and defective in internalization, has been stably expressed in mouse B82L cells [15]. To investigate whether this mutant EGF-R can associate with AP-2, we used both procedures described above. Western-blot analysis of the immunoprecipitates showed that, after EGF activation, the truncated receptor did not recruit detectable amounts of AP-2 complexes (Fig. 1, lanes 22–28). Comparison with the immunoprecipitate

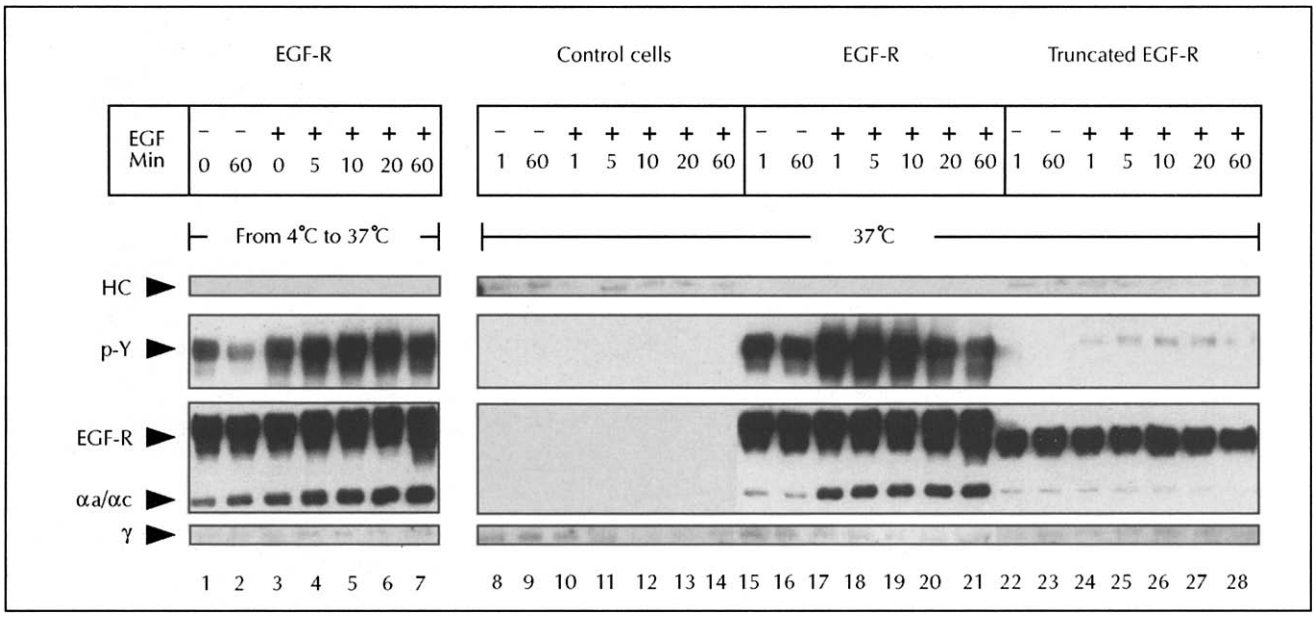


**Fig. 1.** Recruitment of AP-2 complexes by EGF-R *in vivo* requires the regulatory domain of the receptor. Lysates from non-expressor mouse B82L cells (Control cells) and from transfected B82L cells expressing the intact receptor (EGF-R) or the truncated c'958 receptor (Truncated EGF-R) lacking its regulatory domain, were immunoprecipitated with the anti-EGF-R antibody, 13A9, and subjected to SDS-7.5 % polyacrylamide gel electrophoresis (PAGE). Western-blot analysis was carried out using the monoclonal antibodies CHC5.9 specific to clathrin heavy chain (HC), 4G10 specific to phospho-tyrosine (p-Y) and 291-3A specific to EGF-R, and the polyclonal antibodies C6 and C8 directed against the AP-2 αa and αc chains, respectively, and anti-γ directed against the AP-1 γ chain. The lanes represent a similar number of receptors. Lanes 1–7, cells expressing intact receptor were chilled to 4 °C in the (lanes 1,2) absence or (lanes 3–7) presence of 250 ng $ml^{-1}$ EGF, and then transferred to 37 °C for the indicated times. This experiment is representative of 10 carried out. Lanes 8–28, control cells and cells expressing the intact or truncated receptor were kept at 37 °C and incubated in the (lanes 8,9,15,16,22,23) absence or (lanes 10–14,17–21,24–28) presence of 250 ng $ml^{-1}$ EGF for the indicated times. The results are representative of four experiments.

samples from control cells (Fig. 1, lanes 8–14) indicated that there was a small amount of α signal constitutively associated with the truncated receptor. This association may reflect some interaction between AP-2 complexes and the remaining portion of the cytoplasmic tail of the truncated EGF-R. Alternatively, it may indicate an interaction of AP-2 with other proteins that co-immunoprecipitate with EGF-R. For example, a protein of ~190 kDa (probably erb2/neu) becomes tyrosine-phosphorylated in response to EGF and is found in co-immunoprecipitates with EGF-R [14]. From our experiments *in vivo*, we conclude that the regulatory domain is important for the EGF-dependent interaction of EGF-R with AP-2.

The data in Figure 1 also show that, in the mouse cells, AP-1 is not recruited by activated EGF-R (probed for using an anti-γ antibody). Given the sensitivity of the western blots, a low level of association is possible, but it would be less than ~0.1 % of the cellular pool of AP-1 complexes. In agreement with published results [12], clathrin was not recruited by the activated EGF-R (even though the receptor was associated with AP-2 complexes), suggesting that the immunoprecipitates might derive from a step that precedes coated-pit formation. Under the buffer conditions used for immunoprecipitation, clathrin and APs not only remain attached to coated vesicles but also interact efficiently with each other to form coats (our unpublished observations) — any clathrin associated with EGF-R–AP-2 would not have been lost on isolation.

AP-2 complexes contain either an αa or an αc large chain [29]. Western-blot analysis of the immunoprecipitates of EGF-R and of APs from aliquots of EGF-activated cell extracts indicated no preferential interaction of

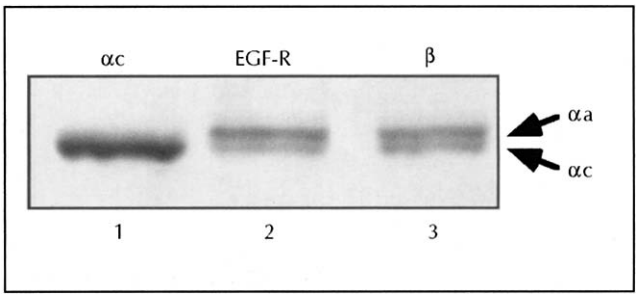


**Fig. 2.** AP-2 complexes recruited by EGF-R *in vivo* contain αa and αc chains. Mouse B82L cells expressing intact EGF-R were incubated at 4 °C with 250 ng $ml^{-1}$ EGF and then transferred for 10 min to 37 °C. The cells were returned to 4 °C, lysed with TGH buffer and the lysates divided into aliquots for immunoprecipitation, SDS-7.5 % PAGE and western-blot analysis. Immunoprecipitates were obtained with the serum 31 directed against the αc chain of AP-2 (lane 1), with the antibody 13A9 against EGF-R (lane 2), and with the serum 32 against the β1 and β2 chains of the AP-1 and AP-2 complexes (lane 3). The membrane was immunoblotted with the antibody AC1-M11 specific for the αa and αc chains of AP-2.

EGF-R with either the αa or the αc class of AP-2 complexes. Both α chains were present in AP-2 bound to EGF-R (Fig. 2, lane 2), although the ratio of αa and αc might be slightly different than the total cellular sampling of AP complexes (Fig. 2, lane 3). The absence of αa signal in the immunoprecipitate with the anti-αc serum (Fig. 2, lane 1) confirmed that only one type of α chain is present in a given AP-2 complex. We conclude that activated EGF-R binds AP-2 selectively *in vivo*, but with no preference for αa or αc.

### Isolated EGF-Rs preferentially associate with purified AP-2 and not with AP-1 *in vitro*

Can the specific association between EGF-R and AP-2 complexes detected *in vivo* be reproduced *in vitro* using isolated molecules? We chose to use surface plasmon resonance (SPR) detection experiments performed in real-time with the BIAcore™ apparatus to develop an *in vitro* method for detecting such interactions. EGF-Rs from a Triton X-100 extract of the transfected mouse B82L cells were adsorbed to the surface of a BIAcore™ sensor chip that contained chemically cross-linked monoclonal antibody 13A9 — specific for the extracellular domain of the receptor. The recruitment of the receptors onto the surface of the sensor chip, monitored as an increase of resonance units (RU), was found to be completely dependent on the presence of the anti-EGF-R antibody (Fig. 3a–d). The binding reached steady-state after 1200 seconds and was saturable — there was no further increase in the signal after another injection of lysate (data not shown). Given that the RU signals are proportional to the mass of protein bound to the sensor chip, and that the antibody molecules are dimeric, we estimate that one molecule of EGF-R monomer was bound per four molecules of divalent antibody. This figure is likely to represent an overestimate of the antibody-binding capacity of the chip, as we did not take into account the added mass of EGF-R due to its glycosylation or to the Triton X-100 micelle attached to it. A similar lysate, obtained from control cells, generated a much smaller signal (less than 5 %; Fig. 3a).

To regenerate the surface of the sensor chip and to restore the capacity of the chip to adsorb fresh EGF-R, bound EGF-Rs were eluted from the cross-linked antibodies with a brief flush of high pH solution containing a small amount of SDS. As shown in Figure 3e, the signal returned to the level seen before the recruitment of EGF-R, and several cycles (10–20) could be carried out before the receptor-binding capacity of the chip declined significantly. This procedure of specific retention, starting from a crude cell extract, has, to our knowledge, not been used before. It provides, in effect, a single efficient step of purification.

Full-length human EGF-R immobilized in this way specifically recruited purified bovine AP-2 complexes (Fig. 4a). Evidence to support the specificity of this association included the lack of AP-2 binding in the absence of EGF-R (Fig. 4a), and other controls such as the lack of BSA binding to immobilized EGF-R and the failure of BSA to compete with AP-2 binding to EGF-R when simultaneously presented to the receptor at ~2 mg $ml^{-1}$ (data not shown). The results from this experiment were

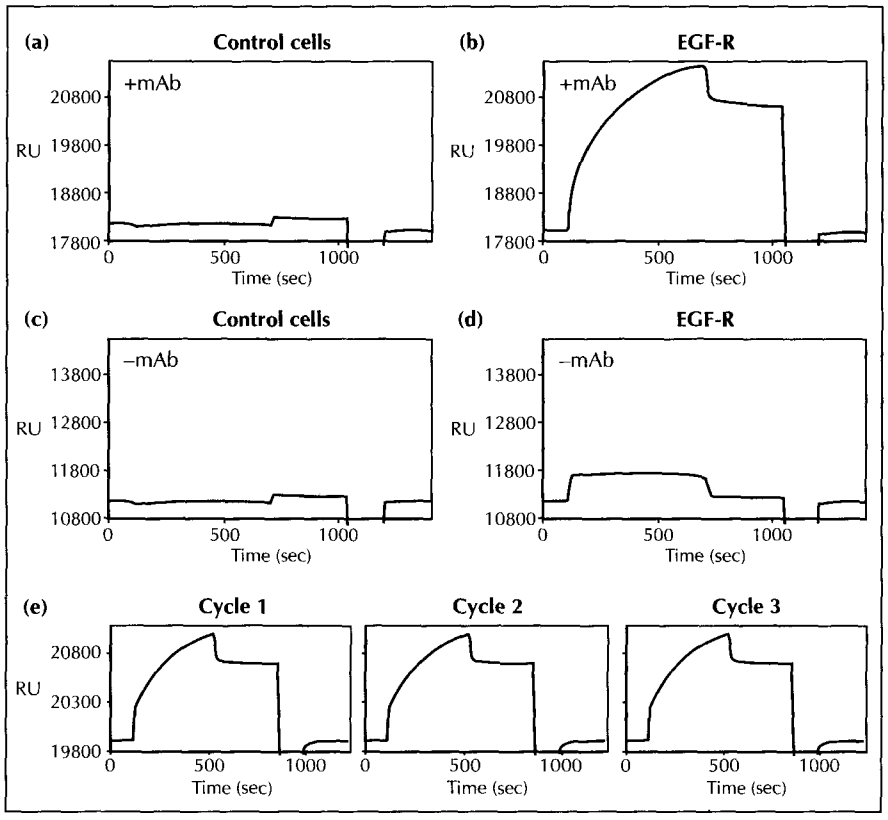


**Fig. 3.** Detection of EGF-R from cell lysates by real-time SPR. **(a–d)** Specificity of receptor recruitment. Cell lysates from (a,c) control B82L cells and (b,d) B82L cells that express intact EGF-R were injected through a sensor chip (a,b) containing immobilized antibody 13A9 specific for the extracellular domain of EGF-R or (c,d) in the absence of antibody. The surface plasmon resonance units (RU) were recorded as a function of time (sensogram). The sharp change in RU at the beginning and end of the sample injection reflects changes in refractive index between the sample and flow buffer. (b) ~2700 RU of EGF-R were captured by the immobilized antibody; (a,c,d) in the control experiments only 100 RU were retained. At ~1000 seconds, bound proteins including APs and EGF-R were released by a flow of 10 mM NaOH, 0.5 % SDS. Similar non-specific association was obtained with the truncated receptor (data not shown). **(e)** Regeneration of the sensor chip. The sensograms correspond to three consecutive cycles of EGF-R capture by the sensor chip from a lysate of B82L expressor cells, followed by their complete release (regeneration) using a short pulse of 10 mM NaOH, 0.5 % SDS starting at ~850 sec. A total of 800 RU were bound and released in each cycle.

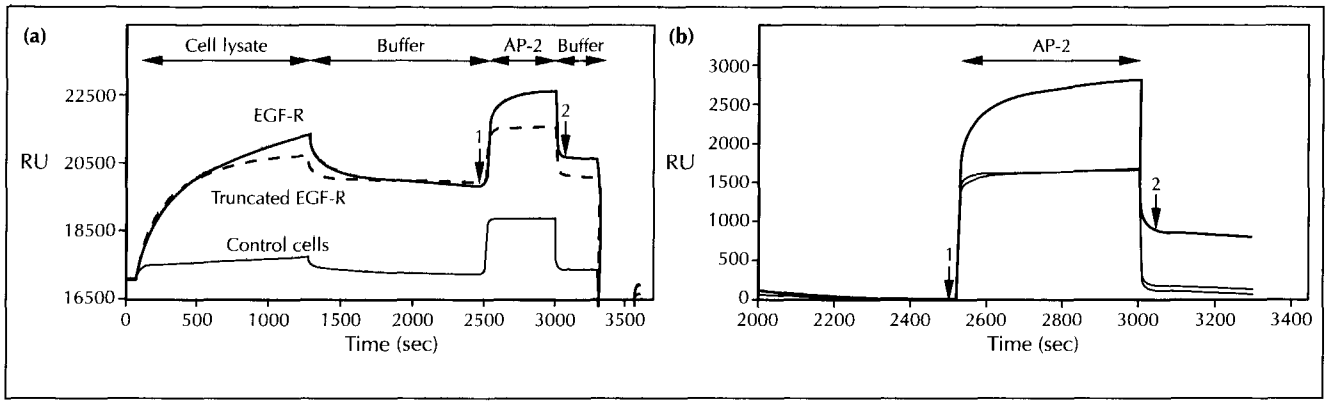


**Fig. 4.** The regulatory domain of isolated EGF-R is required for the interaction of the receptor with purified AP-2 complexes. **(a)** Overlay plot of three sequential sensograms. Firstly, intact EGF-R was captured on the sensor chip. Injection of the cell lysate was followed by a 1200 sec wash with extraction buffer and a 480 sec injection of 32 μl purified AP-2 complexes (0.3 mg $ml^{-1}$; flow of 4 μl $min^{-1}$). After a final 300 sec wash with extraction buffer, the sensor chip was subjected to regeneration, to remove bound EGF-R and APs, as described in Fig. 3. The sharp vertical rises at the beginning and the end of the injections reflect a change in refractive index due to differences in buffer composition. The relative responses corresponding to specific AP-2 association were recorded as the difference in RU reading before (arrow 1) and after (arrow 2) the AP-2 injections. The second sensogram was started by the capture of truncated EGF-R, followed by a cycle of AP-2 injection and regeneration. The third sensogram was recorded with a lysate from control cells. **(b)** The expansion of the overlay plot reveals that AP-2 complexes associate with intact EGF-R but not with the truncated EGF-R or with the lysate from control cells.

reproducible and were repeated several times using independent sensor chips, with different AP-2 preparations and with extracts from several transfected cell lines that overexpress EGF-R — mouse B82L, CHO and Sf9 insect cells (summarized in Fig. 5) and human carcinoma A431 cells (data not shown).

It is likely that most of the detected signal represents AP-2 directly bound to EGF-R rather than through an intermediate protein. Three observations support this deduction. Firstly, the ~170 kDa band of the receptor was by far the most prominent one revealed by SDS-PAGE and Coomassie-Blue staining of EGF-R immunoprecipitates obtained using the same lysis and buffer conditions (data not shown). Secondly, EGF-R produced in transfected insect Sf9 cells also recruited AP-2. It would be necessary to argue that these cells, which overexpress human EGF-R at very high levels, also contain

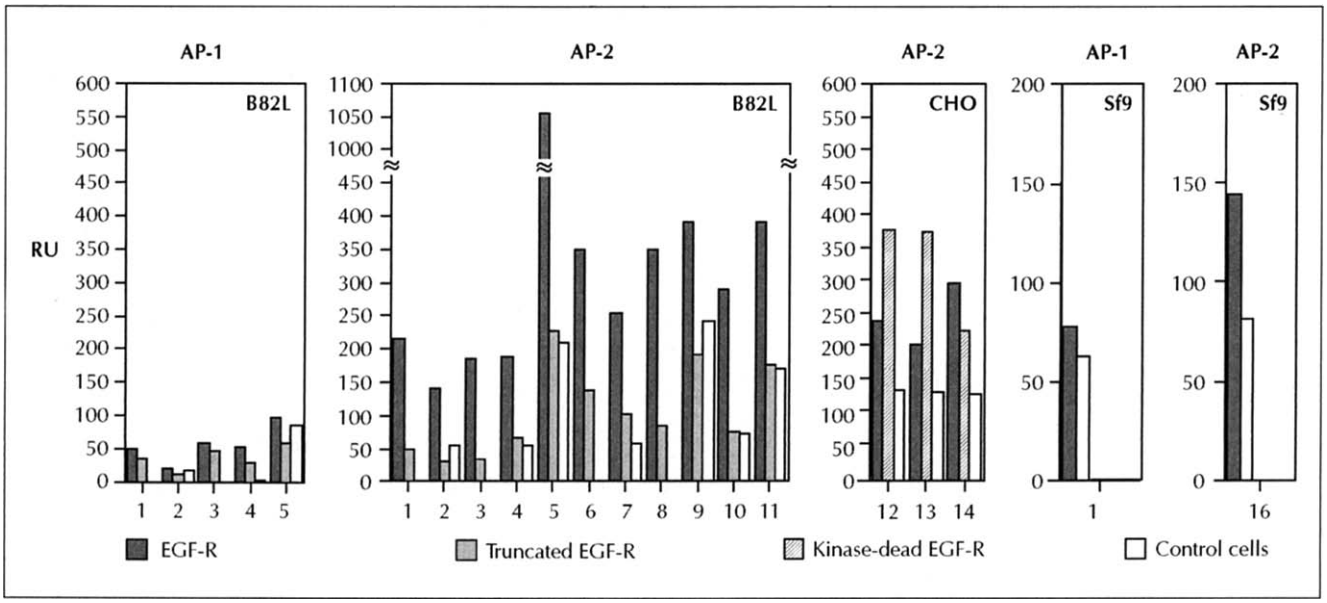


**Fig. 5.** Relative association of AP-1 and AP-2 to intact and mutant EGF-Rs. The bars represent RU readings for bound AP-1 or AP-2 complexes to intact EGF-R, truncated EGF-R and kinase-dead EGF-R as monitored by SPR in 16 independent experiments. Intact EGF-R was expressed in B82L, CHO and Sf9 insect cells. The truncated and kinase-dead receptors were expressed in B82L and CHO cells, respectively. The amount of receptor bound to the sensor chip ranged between 1630–2850 RU. Within individual experiments, RU readings for AP-1 and AP-2 were adjusted by the ratio in molecular weight of the intact and the truncated receptor. The lysates for experiments 1–4 were from cells stimulated with 250 ng $ml^{-1}$ EGF; remaining lysates were from non-stimulated cells. Individual experiments were performed with independent AP-1/AP-2 preparations: experiments 1-3, at ~0.3 mg $ml^{-1}$; 4–6,15,16, at ~0.3 mg $ml^{-1}$; 7, at ~0.3 mg $ml^{-1}$; 8, at ~1 mg $ml^{-1}$; 9-13, at ~1 mg $ml^{-1}$; 14, at ~1 mg $ml^{-1}$.

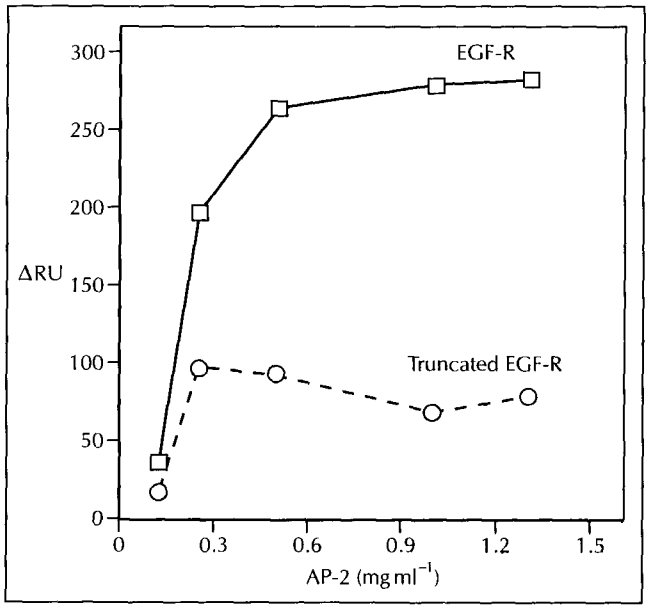


**Fig. 6.** Binding of purified AP-2 complexes to isolated EGF-R is saturable. Purified AP-2 complexes at different concentrations were tested for binding to similar amounts of intact EGF-R and truncated EGF-R receptor bound to the sensor chip. Specifically bound AP-2 is expressed as ΔRU. This value is the difference in RU reading before and after injection of AP-2s (arrows 1,2 in Fig. 4) corrected for the small variation (< 10 %) in the amount of bound receptor in each experiment, for the constant release (3–10 U $min^{-1}$) of bound receptor from the antibody and for the non-specific interaction (< 5 %) obtained with the control cell lysate.

such a putative adaptor molecule. Thirdly, *in vivo*, AP-2 is in direct contact with EGF-R with an approximate 1:1 stoichiometric relationship.

The rates of association and dissociation for AP-2 and the immobilized receptor were noticeably different. The association phase approached steady-state values after 180–360 seconds of AP-2 injection, and the association-rate constant ($k_{on}$) was in the range of $1–3 \times 10^3$ $M^{-1}$ $sec^{-1}$ using AP-2 concentrations up to 1.3 mg $ml^{-1}$ (~5 μM). By comparison, the capture of EGF-R by the immobilized antibody had a $k_{on}$ in the range of $10^5–10^6$ $M^{-1}sec^{-1}$. The release of bound AP-2 complex was too slow to be measured. In contrast, EGF-R recruited by the antibody dissociated with an estimated half-time of 10–15 hours (data not shown). Tight association is also displayed by AP-2 complexes in immunoprecipitates of EGF-R obtained from lysates of cells incubated with EGF before disruption, where the amount of AP-2 co-precipitating with EGF-R is the same 20 minutes or 4 hours after lysis (data not shown and [13]).

Under the conditions used, recruitment of AP-2 by EGF-R reached saturation at ~1.0 mg $ml^{-1}$ AP-2 (Fig. 6), and up to approximately one mole of AP-2 complexes were captured per six moles of immobilized EGF-R. These values are similar to the recruitment of one mole of EGF-R by four moles of chemically immobilized antibody (see above). The 1:4 to 1:6 ratios appear to reflect orientation and accessibility effects when such large molecules are bound to the dextran coating of a sensor chip.

As EGF-R shows selective association with AP-2 over AP-1 *in vivo*, we asked whether this differential selectivity would also be maintained *in vitro* using isolated molecules. Purified AP-1, in the same amount and concentration as AP-2, was used to test its capacity to interact with full length EGF-R. The amount of AP-1 specifically retained by EGF-R in the sensor chip was negligible and similar to the amount which bound to lysates of control cells (Figs 5,7). This result is consistent with the minimal association of AP-1 and EGF-R observed *in vivo* (Fig. 1). The preferential binding of AP-2 detected by SPR is a good indication that the observed association is specific, because the biochemical properties of AP-1 and AP-2 are otherwise very similar (see [3]).

### The regulatory domain of EGF-R is required for association of the receptor with purified AP-2 complexes *in vitro*

As the truncated receptor c'958 is markedly impaired in its ability to associate with AP-2 complexes *in vivo*, we

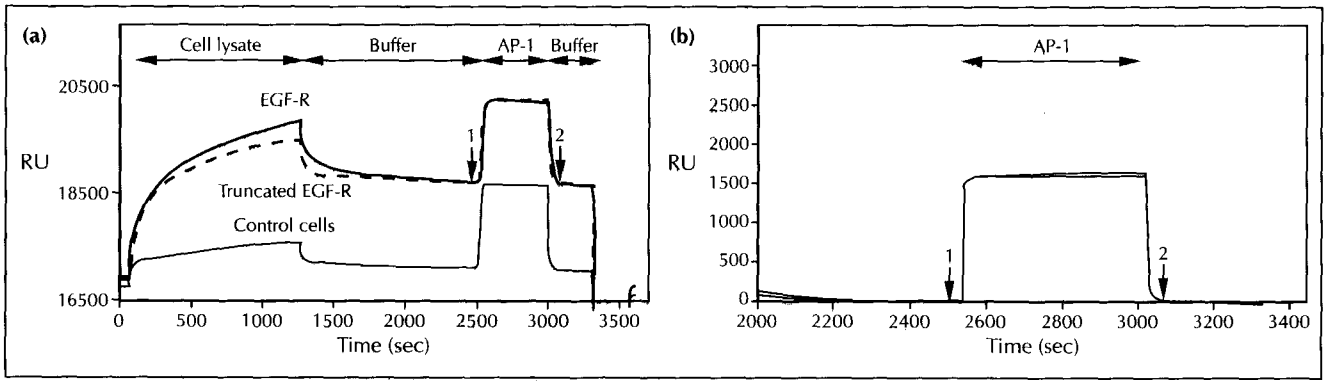


**Fig. 7.** Interaction between AP-1 and isolated EGF-Rs is weak. **(a)** Overlay plot of three sensograms, obtained as described in Fig. 4 and representative of five other experiments. Similar amounts of intact EGF-R and truncated EGF-R were used to probe the interaction with purified AP-1 complexes (0.3 mg $ml^{-1}$). **(b)** The expansion of the overlay plot illustrates the absence of detectable association of AP-1 with the intact EGF-R.

expected that it would also fail to interact with purified AP-2 *in vitro*. Experiments performed using SPR detection confirmed this prediction (Fig. 5, experiments 1–11). An example of an SPR trace showing the absence of specific association between purified AP-2 complexes and the truncated receptor is shown in Figure 4. The extent of binding was comparable to the association detected with the lysate of control cells. Furthermore, no significant association between the truncated receptor and purified AP-1 complexes was detected (Fig. 7). The regulatory domain of the EGF-R is therefore required, both *in vivo* and *in vitro*, for the selective recruitment of AP-2 complexes.

### Phosphorylation of EGF-R is not essential for association with AP-2

It has been proposed, based on endocytosis experiments, that upon autophosphorylation, the cytoplasmic tail of EGF-R undergoes a conformational change and that this change results in the exposure of endocytic signals, leading to internalization of the receptors [22]. Sorkin and Carpenter [12] found, however, that EGF-R from cells blocked in endocytosis due to $K^+$ depletion [30–32] recruits AP-2 even in the absence of EGF activation and EGF-R autophosphorylation. This result suggests that autophosphorylation of the receptor may not be essential for AP-2 recruitment. To test this possibility *in vitro*, we compared the capacity of AP-2 to bind EGF-R with different levels of autophosphorylation obtained by pre-treating the cells with EGF prior to lysis. We found that activation with EGF had no effect on the level of AP-2 binding (Fig. 5, experiments 1–4). We also increased the phosphorylation of EGF-R after capture on the sensor chip, by the addition of 5 mM $Mn_2Cl_2$ and 1 mM ATP to activate the receptor kinase [33,34], or decreased the phosphorylation by the addition of calf-intestine alkaline phosphatase [35]. No effect on the binding of crude APs or of purified AP-2 was detected, even though the receptor phosphorylation varied — monitored by the differential binding to an antibody specific for phospho-tyrosine (data not shown).

While this paper was being revised, Nesterov and colleagues [35] published experiments on the association of EGF-R with crude APs, using an SPR approach similar to the one described here. In their work, they found that phosphorylation of EGF-R *in vitro* correlated with increased AP binding. The conditions of their experiments were different from ours: for example, APs tend to self-associate in the buffer they used. In any case, we demonstrate below that phosphorylation is not essential for AP recruitment by EGF-R *in vivo*. We therefore believe that the characteristics of the isolated receptor studied here correspond well to its properties in a physiological context.

As a complement to the experiments described above, we asked whether a kinase-dead receptor, unable to autophosphorylate, would nevertheless recognize AP-2. To answer this question, we compared the association *in vitro* of AP-2 with wild-type EGF-R and with the EGF-R R721 mutant lacking phosphorylation activity due to the conservative substitution Lys→Arg[721] in its kinase domain [24]. We found that this EGF-R mutant bound AP-2 just as well as wild-type EGF-R (Fig. 5, lanes 12–14).

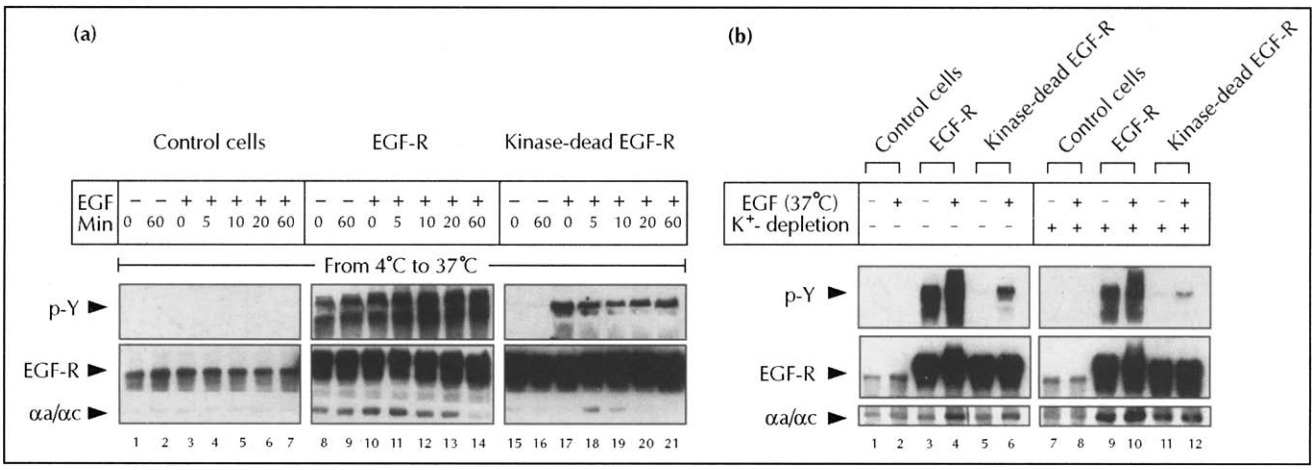


**Fig. 8.** Detection of AP-2 complexes associated with kinase-dead EGF-Rs *in vivo*. **(a)** Lysates from control CHO cells and from transfected CHO cells expressing intact EGF-R or the point mutant R721 with no kinase activity (kinase-dead EGF-R) were immunoprecipitated with the anti-EGF-R antibody 13A9 and subjected to SDS-7.5 % PAGE. The western-blot analysis, representative of three experiments, was done with antibodies against phosphotyrosine, EGF-R and the AP-2 αa and αc chains. The cells were incubated at 4 °C in the absence (lanes 1,2,8,9,15,16) or presence (lanes 3–7,10–14,17–21) of 250 ng $ml^{-1}$ EGF for 60 min and then transferred to 37 °C for the indicated times. **(b)** Cells were left untreated (lanes 1–6) or depleted of $K^+$ (lanes 7–12) for 30 min at 37 °C. Incubation of the cells without (lanes 1,3,5,7,9,11) or with (lanes 2,4,6,8,10,12) 250 ng $ml^{-1}$ EGF for 10 min at 37 °C was followed by receptor immunoprecipitation, SDS-7.5 % PAGE and western blot analysis. The relative strengths of the phosphotyrosine signals were: (lanes 1–6) 0, 0, 23, 67, 1 and 2 %, respectively; (lanes 7–12) 0, 0, 51, 35, 1, and 1.7 %, respectively. The relative strengths of the αa/αc signals were: (lanes 1–6) 2, 6, 11, 34, 18 and 29 %, respectively; (lanes 7–12) 2, 2, 22, 43, 12 ,and 19 %, respectively. These values were obtained by incubation with the appropriate primary antibodies followed by $I^{125}$–protein A and Phosphorimager analysis.

We also studied, *in vivo*, the capacity of the kinase-dead EGF-R mutant to interact with AP-2 in transfected CHO cells (Fig. 8). The results from the temperature-shift experiment (Fig. 8a, lanes 15–21) demonstrate that the kinase-dead EGF-R can indeed associate with AP-2 in response to EGF. However, the strength of the AP-2 signal was approximately 6–8 fold lower than with the wild-type receptor (Fig. 8a, lanes 8–14). In contrast, AP-2 associated with the intact and the kinase-dead receptor to almost the same degree when the cells were kept at 37 °C (sampled after 10 minutes of EGF incubation; Fig. 8b, lanes 4,6). The phosphorylation signal, however, was approximately 28 fold higher for the intact receptor that it was for the sample containing the kinase-dead receptor. It is therefore unlikely that the phosphotyrosine cross-reactive band seen with the kinase-dead receptor can account for the observed AP-2 association. The requirement for active kinase can also be circumvented by $K^+$ depletion even in the absence of EGF (Fig. 8b, lanes 3–6). Thus, depending on the conditions used *in vivo*, there is an efficient association of AP-2, even when the kinase activity of EGF-R is not intact.

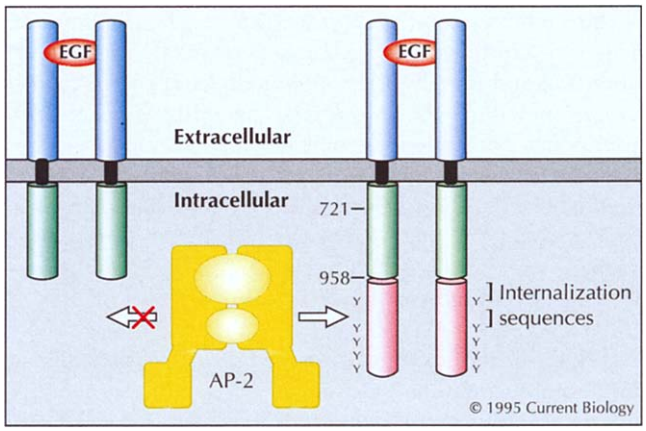


**Fig. 9.** Representation of the interaction of AP-2 with EGF-R. AP-2 association with EGF-R requires the regulatory domain of the receptor. In this model, their interaction precedes the recruitment of clathrin by AP-2 which is necessary to form the coated pit. AP-2 contains the large chains α and β2, with the medium chain μ2 and the small chain σ2 positioned within the AP-core. EGF-R contains on its cytoplasmic tail the kinase domain followed by the regulatory domain. The three known endocytic sequences of EGF-R are located in the regulatory domain and are distinct from the tyrosine auto-phosphorylation sites.

## Discussion

The co-immunoprecipitation experiments and *in vitro* binding studies using SPR reported in this paper show that EGF-R associates preferentially with AP-2 and not with AP-1. The *in vitro* association, therefore, reproduces the specificity observed *in vivo*. The interaction between EGF-R and AP-2 requires the regulatory domain of EGF-R (Fig. 9). Earlier work *in vivo* showed that the regulatory domain of EGF-R is required for EGF-induced internalization from the cell surface [15]. The correlation reported here shows that the regulatory domain of the receptor is essential for the interaction with AP-2, as the truncation of the receptor to residue 958, which abolishes its EGF-induced internalization, also abolishes its capacity to interact with AP-2. Similar immunoprecipitation results were obtained recently with EGF-R truncated to residue 973, another mutant that also fails to internalize in response to EGF [13,35]. Likewise, immunoprecipitation of the related neu proto-oncogene product HER2, a constitutively internalized cell-surface tyrosine kinase, demonstrates an association with AP-2 *in vivo* [36]. The portion distal to the kinase domain is required, as its removal abolishes both internalization and AP-2 interaction. Therefore, it could be argued that the loss of ability of the receptors to interact with AP-2 accounts for this failure to endocytose.

At least three different sequences in the regulatory domain of EGF-R have been shown to serve as internalization signals for this receptor [15]; it remains to be determined whether all participate in independent AP interactions. None of these signals bear a strong resemblance to those in other receptors such as the so-called 'NPXY motif', in which a conserved tyrosine is critical for endocytosis (see [1]). This diversity of internalization signals suggests a multiplicity of targets. There seems, however, to be little heterogeneity in the AP-2 complex itself. Although there are two closely related α chains [29], we have detected no preferential association of either class of AP-2 with EGF-R.

AP complexes may bear a number of distinct sites for interaction with receptor tails, perhaps on more than one polypeptide chain. At least one such site appears to be located in the AP core, the portion of the complex that contains the amino-terminal portions of the α and β chains, together with the medium and small chains [37,38]. AP-2 cores generated by limited proteolysis of EGF-R–AP-2 immunoprecipitates from cell lysates remain associated with EGF-R [12]. Moreover, AP-2 cores are retained on a column containing the recombinant tail of lysosomal acid phosphatase, a lysosomal enzyme trafficked in coated vesicles [11]. It has also been demonstrated that the amino-terminal portion of isolated β2 chains of AP-2 interact with the tail of the asialoglycoprotein receptor [10], and that recombinant α and β2 chains of AP-2 can also interact with EGF-R [35]. There is recent evidence, using the yeast two-hybrid system, that isolated μ1 and μ2, the medium chains of AP-1 and AP-2, interact with the six-residue, tyrosine-based internalization signal of TGN-38 — a protein normally resident in the TGN, which traffics between the plasma membrane and the TGN [39]. Clearly, more studies will be necessary to define the details of these interactions, but it is possible that multiple interactions, each relatively weak, could permit combinatorial regulation of receptor traffic.

What is the role of the EGF-R kinase activity in the recruitment of AP-2? It has been proposed that phosphorylation of the receptor itself acts to regulate endocytosis of EGF-R directly, by inducing a conformational change

in the carboxy-terminal part of the regulatory domain [15]. This conformational change is postulated to relieve inhibition and thereby allow presentation of the internalization signals to the endocytic apparatus. This model appears to be supported by the observation that the EGF-dependent association of EGF-R with AP-2 *in vivo* is enhanced by receptor kinase activity (our data and [35]) as well as by presence of the tyrosine autophosphorylation sites [35], which are distinct from the non-phosphorylated internalization signals [14,15].

A number of other observations indicate, however, that a simple conformational change may not be the only significant event for AP-2 recruitment. It is possible to uncouple the association of EGF-R with AP-2 from the phosphorylation state of the receptor, both *in vivo* and *in vitro* ([12,13] and this work). We have shown that the kinase-dead receptor retains a capacity to recruit AP-2 in an EGF-dependent manner *in vivo*; this recruitment can be almost as high as with the wild-type receptor. Cells depleted of $K^+$, a condition known to retain AP-2 at the plasma membrane but to inhibit EGF-R endocytosis [12,32], show clear association of AP-2 with wild-type and with kinase-dead EGF-R. This association is detected even in the absence of EGF and hence of EGF-induced receptor dimerization and phosphorylation. Furthermore, we show here by SPR that variations in the level of phosphorylation (induced either by pre-treatment of cells with EGF before lysis or by using the kinase-dead EGF-R) have no systematic effect on the extent of AP-2 recruitment by EGF-R. Likewise, *in vitro* phosphorylation or dephosphorylation of EGF-R by treatment with $Mn^{2+}$/ATP or calf-intestine alkaline phosphatase had no detectable effects on its interaction with AP-2 (data not shown). Taken together, these results suggest that the behavior of the isolated receptor resembles that found *in vivo*, including under conditions of $K^+$ depletion. Moreover, they suggest that a simple change in tail conformation is not sufficient to explain the increase in AP-2 affinity displayed by EGF-R upon activation with EGF.

Our data are consistent with the alternative model that invokes an indirect effect of the receptor kinase activity on the regulation of EGF-R endocytosis. In this model, the receptor kinase acts indirectly by the modification of an internalization component, which stimulates endocytosis by facilitating transfer to the endocytic apparatus, presumably AP-2 [23]. Two sets of additional results support this view. First, certain EGF-R mutants, from which the inhibitory domain (including all the auto-phosphorylation sites) has been deleted, still show EGF-dependent endocytosis if their kinase activity is intact. In contrast, these mutants fail to internalize in response to EGF if they also lack kinase activity [14,15]. Second, recent data on the endocytosis of the insulin, transferrin and asialoglycoprotein receptors [27] have demonstrated that a an additional component(s) is required (probably not APs) and that the activity of this component is influenced by the level of cellular kinases [25–27].

What stage of internalization is represented in the co-immunoprecipitate of EGF-R and AP-2? We have confirmed the observation [12] that there is no detectable clathrin in the EGF-R–AP-2 complex and we therefore suggest that co-immunoprecipitation detects a step of association prior to the attachment of clathrin by AP-2 and the formation of a coated pit. The buffer conditions used do not lead to coat disassembly and, in fact, they can be used to drive the formation of clathrin–AP-2 coats *in vitro*. Also, as indicated above, EGF-R recruits AP-2 in cells in which endocytosis has been blocked by $K^+$ depletion. In such cells, very little clathrin is detected on the plasma membrane, although AP-2s are still localized there, as in untreated cells [32].

Finally, we believe that the *in vitro* results presented here represent an important technical development in studies concerning the relatively weak interactions between APs and receptor tails bearing internalization signals. Use of SPR should now permit the dissection of the multiple signals and the identification of their individual targets. The ratio of bound AP-2 to immobilized EGF-R (up to 1:6) is a vast improvement over methods such as column-affinity chromatography, where ratios of about 1:10 000 were found using peptide tails of the 46 kDa mannose-6-phosphate receptor and of the lysosomal acid phosphatase precursor [11].

## Conclusions

A specific interaction between the EGF-R and AP-2 can be detected *in vivo* by immunoprecipitation and *in vitro* by SPR. In both cases, the regulatory domain of the EGF-R cytoplasmic tail is required for association. Autophosphorylation is not essential, however, *in vitro* or *in vivo*. We suggest that the requirement of an active receptor kinase for endocytosis, as observed by others, can best be explained by postulating that phosphorylation of a substrate other than EGF-R itself results in stimulation of receptor uptake. One striking property of the AP-2–EGF-R complex is its stability, as followed by SPR — we have not been able to measure dissociation. It is possible that, *in vivo*, a further protein participates in release of the receptor from AP-2.

## Materials and methods

### *Materials*

The monoclonal antibodies AC1-M11 (specific for the αa and αc chains of AP-2) and B1M6 (specific for β1 and β2 of AP-1 and AP-2) and polyclonal antibodies C6 (specific for αa), C8 (specific for αc), and anti-γ (specific for the hinge-region and amino terminus of γ chains) were gifts of M. Robinson (University of Cambridge). The monoclonal antibody 4G10 against phosphotyrosine was a gift of T. Roberts (Dana-Farber Cancer Institute). The monoclonal antibody 13A9 (specific for the extracellular region of the human EGF-R) was a gift of B. Fendly (Genentech). The monoclonal antibody 291-3A (specific for the intracellular domain of the human EGF-R) was

provided by K. L. Carraway III (Harvard Medical School). The rabbit serum 31 (specific for αc chains) immunoprecipitates AP-2 complexes, whereas the serum 32 (specific for β chains) immunoprecipitates both AP-1 and AP-2 complexes [13].

Mouse B82L cells, expressing the intact human EGF-R or the truncated form c'958 lacking its regulatory domain, and the control cells transfected with the dihydrofolate reductase gene were a generous gift of G. Gill (University of California, San Diego). CHO cell lines, expressing the intact human EGF-R or the kinase-dead EGF-R mutant R721 (Lys→$Arg^{721}$) were generous gifts of R. Davis (University of Massachusetts).

Media and bovine sera were from GIBCO; glutamine, methotrexate and bovine serum albumin (BSA; Fraction V) from SIGMA; Serum Plus and antibiotics from JRH Biosciences; recombinant human EGF from UBI; protein A-Sepharose from Pharmacia. Horseradish-conjugated antibodies to mouse IgG (Amersham) and rabbit IgG (Boehringer Mannheim), nitrocellulose membranes (Schleicher & Schuell) and enhanced chemiluminescence reagents (Amersham) were used for immunoblots.

### *Tissue culture*

Cells were cultured at 37 °C, 100 % humidity and 5 % $CO_2$ with 2 mM glutamine and 100 U $ml^{-1}$ penicillin/0.1 mg $ml^{-1}$ streptomycin. Control B82L cells were grown in Dulbeccos Minimal Essential Medium (DMEM) with 5 % calf serum and the human *EGF-R*-transfected B82L cells in DMEM with 5 % dialyzed fetal bovine serum (FBS) and 500 nM methotrexate. CHO-K1 cells (ATCC) were grown in $\alpha^-$-Minimal Essential Medium ($\alpha^-$-MEM), 5 % FBS and 5 % Serum Plus. The transfected CHO cells were maintained in $\alpha^-$-MEM, 10 % dialyzed FBS and 200 μM methotrexate. Before use, the cells were expanded in non-selecting media for one or two passages.

### *Association of EGF-Rs with APs in living cells*

Cells were grown to confluency in 100 mm dishes and serum-starved in DMEM (B82L lines) or $\alpha^-$-MEM (CHO lines) containing 0.1 % BSA for 24 h. EGF-Rs were activated [12] with the following modifications. The dishes were transferred to 4 °C in the same media (pre-chilled) and incubated with ± 100–250 ng $ml^{-1}$ (16–40 nM) human EGF for 60 min followed by transfer to 37 °C for various times in the absence of EGF. Alternatively, cells were kept at 37 °C during stimulation with 250 ng $ml^{-1}$ EGF and then returned to 4 °C by two sequential washes with chilled PBS. Cells were lysed with 0.7 ml TGH lysis buffer (50 mM HEPES pH 7.3, 10 % glycerol, 1 % Triton X-100, 1 mM $Na_3VO_4$, 0.5 mM PMSF, 20 μM leupeptin). After 10 min at 4 °C, the cells were scraped, vortexed and lysates cleared by centrifugation at 4 °C (15 min at top speed in an Eppendorf centrifuge). The $K^+$-depletion experiments were performed at 37 °C [40].

### *Immunoprecipitations*

10 μl $ml^{-1}$ antibody 13A9 (1.6 mg $ml^{-1}$) was added to lysates and incubated with constant inversion at 4 °C for 90 min. 45 μl $ml^{-1}$ protein A Sepharose beads, previously blocked with BSA were then added and incubation continued for another 90 min at 4°C. Alternatively, the same amount of antibody was pre-immobilized to protein A-Sepharose beads in PBS/0.1 % Triton X-100 at 4 °C for 60 min on a rocking platform and, after two washes with chilled PBS, added to the lysates for 60 min at 4 °C. The immunoprecipitates were washed once with TGH and once with TGH/100 mM NaCl, boiled in Laemmli buffer with 2.5 % β-mercaptoethanol and resolved by SDS-7.5 % PAGE. For each experiment, it was verified that all immunoprecipitated samples had the same amount of EGF-R.

### *Immunoblotting*

Proteins, electro-transferred onto nitrocellulose membranes, were probed with primary antibodies by overnight incubation at 4 °C, or 4 h at room temperature. Secondary antibodies, conjugated to horseradish peroxidase, were added for 60 min at room temperature and the signals developed for enhanced chemiluminescence. Several film exposures with the same blot and serial dilutions of the same sample were used to be in the linear range of sensitivity. Specificity and sensitivity of the immunoreactive signals were verified by analysis of cell lysates and purified bovine-brain APs. Additional experiments, not shown here, using $I^{125}$-protein-A to probe the nitrocellulose blots or secondary antibodies conjugated to alkaline phosphatase, after protein transfers onto PVDF membranes, gave identical results.

### *Purification of clathrin-associated protein complexes AP-1 and AP-2*

AP-1 and AP-2 complexes were obtained from crude bovine brain APs [41] by hydroxyapatite ion-exchange chromatography [5] as follows. Crude APs, dialyzed at 4 °C against 10 mM $NaH_2PO_4$ pH 7.1, 100 mM NaCl, 0.1 % β-mercaptoethanol, 0.02 % $NaN_3$, were applied to a 5 ml hydroxyapatite column (Econo-Pac® HTP Cartridge, Bio-Rad) connected to an FPLC system (Pharmacia) equilibrated in the same buffer. AP-1 and AP-2 were resolved at 0.5 ml $min^{-1}$ using a 40 ml gradient ranging from 10–350 mM $NaH_2PO_4$.

### *BIAcore™ amine coupling of antibody*

Surface plasmon resonance (SPR) detection experiments were performed with the BIAcore™ apparatus at 25 °C using CM5-research grade sensor chips (Pharmacia Biosensor). 32 μl antibody 13A9 (80 μg $ml^{-1}$ in 10 mM sodium acetate pH 5.0) at a flow of 5 μl $min^{-1}$ was cross-linked by its primary amine groups to the carboxymethylated dextran surface of the sensor chip according to the manufacturer (Pharmacia Biosensor). The resonance signal increased by 8000–9000 RU (~ 500 nM) [42].

### *Binding of EGF-Rs to antibodies immobilized onto the sensor chip*

Cells were grown to confluency on 150 mm dishes, washed with chilled PBS and lysed with 0.3 ml extraction buffer (20 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1 % Triton X-100 pH 7.4) supplemented with 1 mM $Na_3VO_4$, 0.5 mM PMSF, 1 mg $ml^{-1}$ leupeptin and 1 mg $ml^{-1}$ aprotinin. The cells were scraped and cleared at 4 °C by sequential centrifugation at top speed for 15 min (Eppendorf centrifuge) and at 85 000 rpm for 30 min (100.4 rotor, Beckman TLX). 40 ml cleared lysate was injected into the BIAcore™ apparatus at 2 ml $min^{-1}$. The EGF-Rs only adsorbed to sensor chips containing the anti-EGF-R antibody 13A9. The increase in resonance signal following injection of the lysate of transfected or non-transfected cells was in the range of 1500–3000 RU and 100-300 RU, respectively. Because of dissociation of EGF-Rs from the antibodies, there is a baseline drift in the range of 3–10 RU $min^{-1}$.

### *Injection of purified AP-1 and AP-2 complexes to the sensor chip*

AP-1 or AP-2 complexes (~ 0.3 mg $ml^{-1}$) were supplemented with Triton X-100 to prevent aggregation (0.4 % for AP-1 and 0.1 % for AP-2) and dialyzed overnight against AP-binding buffer (100 mM NaMES, 150 mM NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.02 % $NaN_3$, 0.2 mM DTT, and 0.4 or 0.1 %

Triton X-100 pH 7.0). AP-2s were concentrated 4-5 fold to a final concentration of 1.3 mg $ml^{-1}$ using a Centriprep-30 unit (Amicon). After a high-speed centrifugation step (85 000 rpm, 30 min, 4 °C; 100.4 rotor), 32 µl of the supernatants were injected at a flow of 2–4 µl $min^{-1}$. Binding was determined by the increase in resonance signal at the end of each injection relative to the baseline signal before injection. AP-1 and AP-2 association by lysates from expressor cells was corrected from the signal elicited by the corresponding control cells.

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## References


1. Trowbridge IS, Collawn JF: **Signal-dependent membrane protein trafficking in the endocytic pathway.** *Annu Rev Cell Biol* 1993, **9**:129–161.
2. Robinson MS: **Adaptins.** *Trends Cell Biol* 1992, **2**:293–297.
3. Kirchhausen T: **Coated pits and coated vesicles — sorting it all out.** *Curr Opin Struc Biol* 1993, **3**:182–188.
4. Zaremba S, Keen JH: **Assembly polypeptides from coated vesicles mediate reassembly of unique clathrin coats.** *J Cell Biol* 1983, **97**:1339–1347.
5. Pearse BM, Robinson MS: **Purification and properties of 100 kD proteins from coated vesicles and their reconstitution with clathrin.** *EMBO J* 1984, **3**:1951–1957.
6. Heuser J, Kirchhausen T: **Deep-etch views of clathrin assemblies.** *J Ultrastruct Res* 1985, **92**:1–27.
7. Vigers GP, Crowther RA, Pearse BM: **Location of the 100 kd–50 kd accessory proteins in clathrin coats.** *EMBO J* 1986, **5**:2079–2085.
8. Pearse BM: **Receptors compete for adaptors found in plasma membrane coated pits.** *EMBO J* 1988, **7**:3331–3336.
9. Glickman JN, Conibear E, Pearse BM: **Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor.** *EMBO J* 1989, **8**:1041–1047.
10. Beltzer JP, Spiess M: ***In vitro* binding of the asialoglycoprotein receptor to the beta adaptin of plasma membrane coated vesicles.** *EMBO J* 1991, **10**:3735–3742.
11. Sosa MA, Schmidt B, von Figura K, Hille-Rehfeld A: ***In vitro* binding of plasma-membrane coated-vesicle adaptors to the cytoplasmic domain of lysosomal acid phosphatase.** *J Biol Chem* 1993, **268**:12537–12543.
12. Sorkin A, Carpenter G: **Interaction of activated EGF receptors with coated-pit adaptins.** *Science* 1993, **261**:612–615.
13. Sorkin A, McKinsey T, Shih W, Kirchhausen T, Carpenter G: **Stoichiometric interaction of the epidermal growth-factor receptor with the clathrin-associated protein complex AP-2.** *J Biol Chem* 1995, **270**:619–625.
14. Chen WS, Lazar CS, Lund KA, Welsh JB, Chang C-P, Walton GM, *et al.*: **Functional independence of the epidermal growth-factor receptor from a domain required for ligand-induced internalization and calcium regulation.** *Cell* 1989, **59**:33–43.
15. Chang C-P, Lazar CS, Walsh BJ, Komuro M, Collawn JF, Kuhn LA, *et al.*: **Ligand-induced internalization of the epidermal growth-factor receptor is mediated by multiple endocytic codes analogous to the tyrosine motif found in constitutively internalized receptors.** *J Biol Chem* 1993, **268**:19312–19320.
16. Yarden Y, Schlessinger J: **Phosphorylation of epidermal growth-factor receptor: evidence for a model of intermolecular allosteric activation.** *Biochemistry* 1987, **26**:1434–1442.
17. Margolis BL, Lax I, Dombalagian M, Honegger AM, Howk R, Givol D, *et al.*: **All autophosphorylation sites of epidermal growth-factor (EGF) receptor and HER2/neu are located in their carboxy-terminal tail.** *J Biol Chem* 1989, **264**:10667–10671.
18. Walton GM, Chen WS, Rosenfeld MG, Gill GN: **Analysis of deletions of the carboxyl-terminus of the epidermal growth-factor receptor reveals self-phosphorylation at tyrosine 992 and enhanced *in vivo* tyrosine phosphorylation of substrates.** *J Biol Chem* 1990, **265**: 1750–1754.
19. Sorkin A, Waters C, Overholser KA, Carpenter G: **Multiple autophosphorylation site mutations of the epidermal growth-factor receptor.** *J Biol Chem* 1991, **266**:8355–8362.
20. Lowenstein EJ, Daly RJ, Gatzer AG, Li W, Margolis B, Lammers R, *et al.*: **The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling.** *Cell* 1992, **70**:431–442.
21. Glenney JR, Chen WS, Lazar CS, Walton GM, Zokas LM, Rosenfeld MG, *et al.*: **Ligand-induced endocytosis of the EGF receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies.** *Cell* 1988, **52**:675–684.
22. Cadena DL, Chan C-L, Gill GN: **The intracellular tyrosine kinase domain of the epidermal growth factor receptor undergoes a conformational change upon autophosphorylation.** *J Biol Chem* 1994, **269**:260–265.
23. Wiley HS, Herbst JJ, Walsh BJ, Lauffenburger DA, Rosenfeld MG, Gill GN: **The role of tyrosine kinase activity in endocytosis, compartmentation, and down-regulation of the epidermal growth factor receptor.** *J Biol Chem* 1991, **266**:11083–11094.
24. Countaway JL, Nairn AC, Davis RJ: **Mechanism of desensitization of the epidermal growth-factor receptor protein-tyrosine kinase.** *J Biol Chem* 1992, **267**:1129–1140.
25. Lamaze C, Baba T, Redelmeier TE, Schmid SL: **Recruitment of epidermal growth factor and transferrin receptors into coated pits *in vitro*: differing biochemical requirements.** *Mol Biol Cell* 1993, **4**:715–727.
26. Fallon RJ, Danaher M, Saylors RL, Saxena A: **Defective asialoglycoprotein receptor endocytosis mediated by tyrosine kinase inhibitors; requirement for a tyrosine in the receptor internalization signal.** *J Biol Chem* 1994, **269**:11011–11017.
27. Welsh JB, Worthylake R, Wiley HS, Gill GN: **Specific factors are required for kinase-dependent endocytosis of insulin receptors.** *Mol Biol Cell* 1994, **5**:539–547.
28. Herbst JJ, Opresko LK, Walsh BJ, Lauffenburger DA, Wiley HS: **Regulation of postendocytic trafficking of the epidermal growth factor receptor through endosomal retention.** *J Biol Chem* 1994, **269**: 12865–12873.
29. Robinson MS: **Cloning of cDNAs encoding two related 100 kD coated vesicle proteins (alpha-adaptins).** *J Cell Biol* 1989, **108**: 833–842.
30. Larkin JM, Brown MS, Goldstein JL, Anderson RG: **Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts.** *Cell* 1983, **33**:273–285.
31. Heuser JE, Anderson RG: **Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation.** *J Cell Biol* 1989, **108**:389–400.
32. Hansen SH, Sandvig K, van Deurs B: **Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification.** *J Cell Biol* 1993, **121**:61–72.
33. Wedegaertner PB, Gill GN: **Activation of the purified protein tyrosine kinase domain of the epidermal growth factor receptor.** *J Biol Chem* 1989, **264**:11346–11353.
34. Mohammadi M, Honegger A, Sorokin A, Ullrich A, Schlessinger J, Hurwitz DR: **Aggregation-induced activation of the epidermal growth-factor receptor protein tyrosine kinase.** *Biochemistry* 1993, **32**:8742–8748.
35. Nesterov A, Kurten RC, Gill GN: **Association of epidermal growth factor receptors with coated-pit adaptins via a tyrosine-phosphorylation-regulated mechanism.** *J Biol Chem* 1995, **270**:1–8.
36. Gilboa L, Ben-Levy R, Yarden Y, Henis YI: **Roles for a cytoplasmic tyrosine and tyrosine kinase activity in the interactions of Neu receptors with coated pits.** *J Biol Chem* 1995, **270**:7061–7067.
37. Zaremba S, Keen JH: **Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity.** *J Cell Biochem* 1985, **28**:47–58.
38. Kirchhausen T, Nathanson KL, Matsui W, Vaisberg A, Chow EP, Burne C, *et al.*: **Structural and functional division into two domains of the large (100 to 115 kDa) chains of the clathrin-associated protein complex AP-2.** *Proc Natl Acad Sci USA* 1989, **86**:2612–2616.
39. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, *et al.*: **Interaction of tyrosine-based sorting signals with the medium chain of clathrin-associated complexes.** *Science* 1995, in press.
40. Larkin JM, Donzell WC, Anderson RG: **Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices.** *J Cell Biol* 1986, **103**:2619–2627.
41. Matsui W, Kirchhausen T: **Stabilization of clathrin coats by the core of the clathrin-associated protein complex AP-2.** *Biochemistry* 1990, **29**:10791–10798.
42. Johnsson B, Lofas S, Lindquist G: **Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors.** *Anal Biochem* 1991, **198**:268–277.


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