The EMBO Journal vol.5 no.9 pp.2179–2190, 1986

Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization

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Communicated by J.Schlessinger

Binding of epidermal growth factor (EGF) to its receptor results in a cascade of events that culminate in cell division. The receptor is present on the cell surface in two forms of high and low affinity binding for EGF. EGF binding activates the receptor's intracellular tyrosine kinase activity and subsequently causes the receptor to be rapidly internalized into the cell via clathrin-coated pits. We have cloned the EGF receptor cDNA into a retroviral expression vector and made mutations *in vitro* to investigate the function of different receptor domains. Deletion of cytoplasmic sequences abolishes high but not low affinity sites as well as impairing the ability of the protein to internalize into cells. Thus, cytoplasmic sequences must be involved in the regulation of high affinity sites and are required for EGF-induced receptor internalization. A four amino acid insertion mutation at residue 708 abolishes the protein–tyrosine kinase activity of the immunoprecipitated receptor. However, this receptor mutant exhibits both the high and low affinity states, internalizes efficiently and is able to cause cells to undergo DNA synthesis in response to EGF. Another four amino acid insertion mutation (residue 888) abolishes protein–tyrosine kinase activity, high affinity binding, internalization and mitogenic responsiveness. Finally, a chimaeric receptor composed of the extracellular EGF binding domain and the cytoplasmic v-*abl* kinase region transforms Rat-I cells. This chimaeric receptor possesses intrinsic protein tyrosine kinase activity which cannot be regulated by EGF. Moreover, EGF fails to induce the internalization of the chimaeric receptor.

Key words: EGF receptor/cDNA/cytoplasmic domain/deletion analysis/internalization

Introduction

Epidermal growth factor (EGF) is a potent mitogenic polypeptide that binds to a specific receptor which is expressed on the surface of various epithelial, epidermal and fibroblastic cells (for reviews see Carpenter and Cohen, 1979; Schlessinger *et al.*, 1983). The EGF receptor (EGF-R) contains a protein–tyrosine kinase activity which is inducible by EGF and which leads to the phosphorylation of various cellular proteins including the self phosphorylation of the EGF-receptor molecule (Ushiro and Cohen, 1980; Hunter and Cooper, 1981). Binding experiments with [^{125}I]EGF indicate that the receptor is present on the cell surface in two forms of high and low affinity for EGF (Schechter *et al.*, 1978; King and Cuatrecasas, 1982; Rees *et al.*, 1984). The relationship and importance of these two forms is not known. After binding, the receptor internalizes rapidly into cells via clathrin-coated pits (reviewed in Schlessinger *et al.*, 1983). Despite elucidation of these early responses it is not clear how they are related to the ability to induce DNA synthesis in as much as EGF must be present in the extracellular medium for >6–10 h to have a mitogenic effect (reviewed in Carpenter and Cohen, 1979 and Schelssinger *et al.*, 1983).

The purification (Yarden *et al.*, 1985) and subsequent cDNA cloning of the EGF receptor resulted in the deduction of the complete primary structure of the protein (Downward *et al.*, 1984; Ullrich *et al.*, 1984). It is comprised of an extracellular cysteine-rich EGF-binding domain, a single hydrophobic transmembrane domain and an intracellular tyrosine kinase domain among other possible structures. Similar cysteine-rich sequences are present in the extracellular sections of low density lipoprotein (LDL) and insulin receptors as well as the precursor of epidermal growth factor (Lehrman *et al.*, 1985; Ullrich *et al.*, 1985; reviewed in Pfeffer and Ullrich, 1985). The sequence also revealed that the EGF receptor is highly related to the v-*erbB* oncogene of avian erythroblastosis virus and is therefore probably equivalent to the proto-oncogenic c-*erbB*. The v-*erbB* product is a truncated form of the EGF receptor, however amplification of the EGF receptor gene has also been associated with a neoplastic phenotype (Ullrich *et al.*, 1984; Merlino *et al.*, 1984; Lin *et al.*, 1984; Libermann *et al.*, 1985).

We have utilized the cDNA clone of the receptor to analyze which sequences of the receptor are responsible for its biological properties. The cDNA has been cloned into a retrovirus expression vector and transfected into NIH/3T3 fibroblast cells. Mutations have been made in the transfected plasmid to study the importance of specific sequences. The ability of cytoplasmic sequences to affect high versus low affinity binding of the extracellular domain, EGF-induced internalization, tyrosine kinase activity and mitogenicity is reported here.

Results

We utilized a murine retrovirus vector, pZIPNeoSV(X) (Cepko *et al.*, 1984), to express the EGF receptor (EGF-R) cDNA. The structure of this plasmid, pER, containing the cDNA is shown in Figure 1a. The cDNA is expressed directly from the retroviral LTR promoter while the Geneticin G418 (neomycin) resistance gene (*Neo*^{R}) is expressed via a sub-genomic message using the natural Moloney murine leukemia virus (M-MuLV) 5′ and 3′ splice sites in the positions indicated in Figure 1a.

The full coding region of the EGF receptor was constructed using clones λHER64 and λHER21 from Ullrich *et al.* (1984). The inserted EGF-R cDNA stretched from an *Sst*I site 20 bases before the initiation codon (AUG) to an *Xmn*I site 150 nucleotides past the termination codon for translation (see Experimental Procedures for details). A minimal amount of the 5′ untranslated region was included so as not to interfere with translational initiation within the framework of the retrovirus message.

The general strategy was to transfect the expression vector into ψ-2 cells and select for cells resistant to neomycin. ψ-2 cells are NIH/3T3 cells transfected with a defective clone of M-MuLV (Mann *et al.*, 1983). These cells allow for the rescue of virus

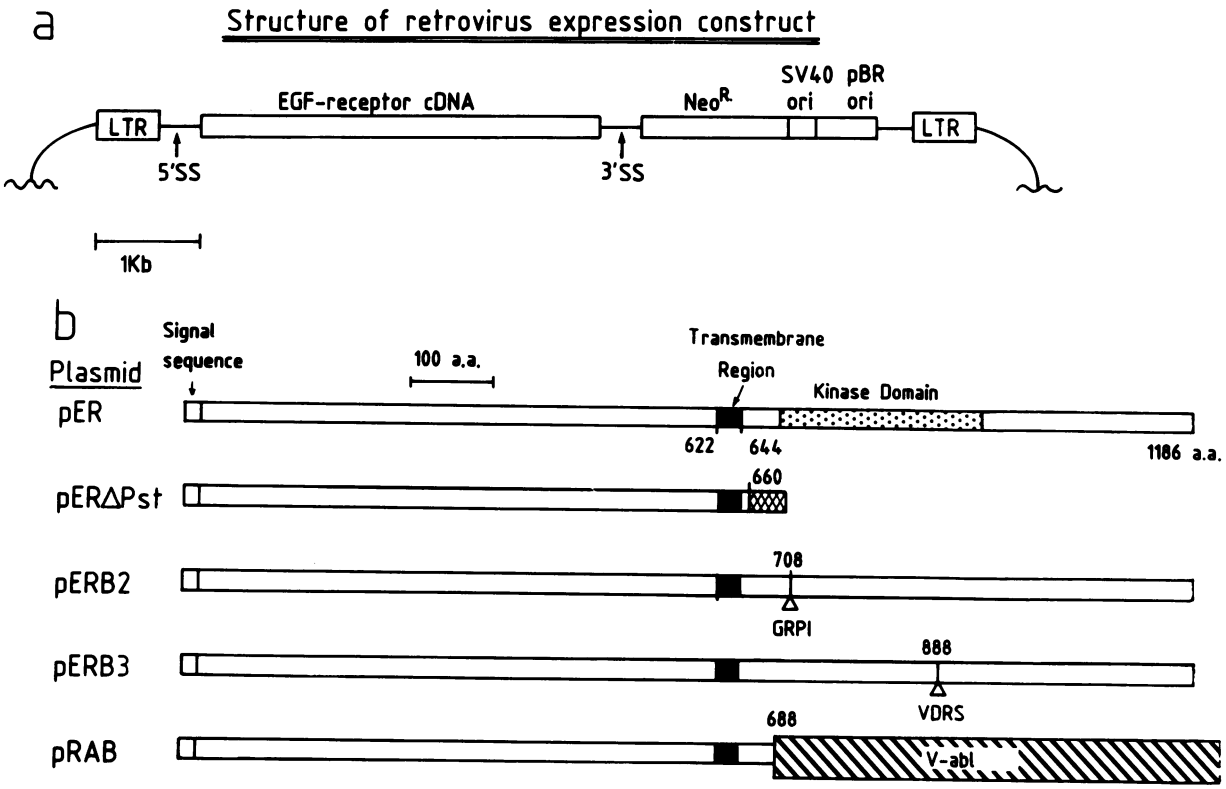

Fig. 1. Structure of retrovirus expression construct and mutants. (a) The structure of plasmid pER is shown. Transcription initiates in the left long terminal repeat (LTR) derived from Moloney murine leukemia virus (M-MuLV) and terminates in the right LTR. The position of M-MuLV 5′ and 3′ splice sites are shown. This splice is used to express the G418 antibiotic resistance gene (Neo^R). The positions of SV40 virus replication origin and pBR322 plasmid replication origin DNA are indicated. (b) The structures of the coding region of mutants are shown. The signal sequence, transmembrane region and kinase domain are indicated. The amino acid numbering is from immediately after the signal sequence to 1186 amino acids for the 'wild type' receptor. The hatched box in pERΔPst indicates sequences translated out of frame. The positions and amino acids inserted for pERB2 and pERB3 are as indicated.

containing the transfected genome, free of the M-MuLV helper virus. We were able to harvest a recombinant virus from ψ-2 cells transfected with pER which passaged both the Neo^R gene as well as the EGF-receptor gene. This virus however was present at very low titers (10^2-10^3 neomycin-resistant colony forming units per ml) most probably due to inefficient splicing to the Neo^R gene when the EGF-R insert is present. Due to these difficulties we have not pursued viral studies using this construct. Nevertheless, the vector was able to efficiently express EGF-R following transfection as demonstrated by the expression of an active EGF receptor (see next section).

Mutants of EGF receptor were constructed *in vitro* in the expression vector pER. The structure of the resultant coding sequences are diagrammed in Figure 1b.

pERΔ Pst. To understand the role of the cytoplasmic part of the EGF-R a deletion was made so as to leave only 16 amino acids following the membrane spanning domain. The coding region then proceeds for 65 amino acids out of frame as predicted from the nucleotide sequence (Ullrich *et.al.*, 1984).

pERB2. Insertion mutants were made in the kinase domain of EGF-R in order to determine the role of the kinase domain on other biological properties of the receptor. Synthetic *Sal*I linker DNA (5′GGTCGACC3′) was inserted at a *Bam*HI site near the beginning of the kinase domain. The method of insertion results in an in-frame insertion of four amino acids (GRPI) after amino acid number 708 as indicated in Figure 1b.

pERB3. Similar to pERB2, a synthetic *Sal*I linker was inserted at another *Bam*HI site near the 3′ end of the kinase domain resulting in an insertion of four amino acids (VDRS) after amino acid 888 as shown in Figure 1b.

pRAB. This is a fusion gene of EGF-R and the kinase domain of v-*abl*. The v-*abl* oncogene of Abelson murine leukemia virus (A-MuLV) encodes a product with protein tyrosine kinase activity similar to that of EGF-R (Witte *et al.*, 1980). This is also reflected in the amino acid homology between each of their kinase domains (Bishop, 1983; Ullrich *et al.*, 1984). We were interested in determining the effect of deletion of the EGF-R sequences on its activities as well as studying whether the external EGF-binding domain of EGF receptor could modulate the activity of an heterologous kinase domain.

Characterization of proteins in transfected cell lines

The plasmid pER, containing 'wild type' human EGF-receptor sequences, was transfected into ψ-2 cells. Colonies of cells resistant to the antibiotic G418 were isolated and found to contain the human EGF-R protein. Transfected, ψER-2, and non-transfected, ψ-2, cells were treated with or without EGF, immunoprecipitated with RK-2 (an anti-peptide antibody which recognizes a region in the cytoplasmic domain of EGF-receptor, Kris *et al.*, 1984) and subjected to the standard autophosphorylation reaction. No activity was detected in ψ-2 cells while an EGF-induced activity was found in ψER-2 (Figure 2). The 170 kd protein from ψER-2 co-migrated with EGF-R from human A-431 carcinoma cells and is precipitable with other EGF-R specific antibodies available to us (data not shown).

The level of expression of EGF-R in ψER-2 was very high as determined by EGF binding experiments (5.7×10^6 recep-

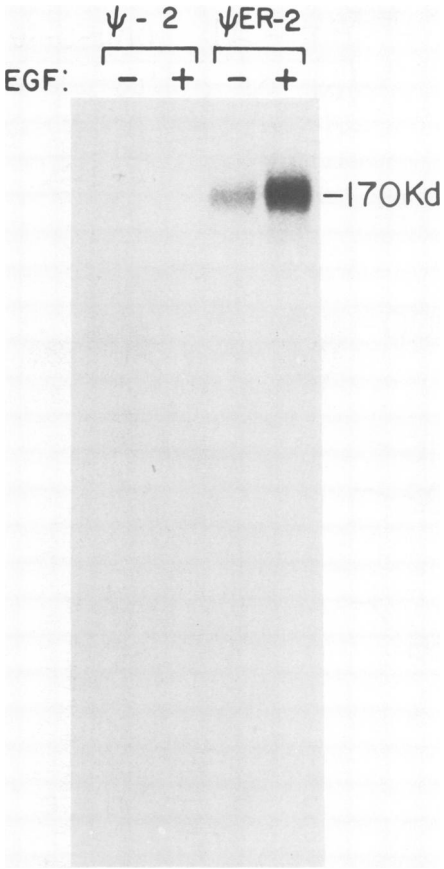

Fig. 2. Autophosphorylation of the transfected EGF receptor gene product. Control (ψ-2) and transfected (ψER-2) cells were solubilized and treated with or without 5 μg/ml EGF for 20 min on ice. EGF-R-specific serum (RK-2) was used to precipitate the receptor and the washed precipitate was incubated with [γ-^{32}P]ATP in an autophosphorylation assay as described in Experimental procedures. The products were analyzed by 7% SDS-PAGE and autoradiography. The position of 170 kd mol. wt as determined from known markers is shown.

tors/cell; Table I) and by the standard autophosphorylation assay. In fact, the expression in ψER-2 was 10- to 50-fold higher than the expression in other cell lines we generated. For a better comparison to normal mitogenically responsive cells and to cell lines bearing mutant transfected receptors we sought a cell line with a more similar number of receptors. During the course of our studies of virus expression we produced a cell line, N22, using virus from ψER-2 to infect NIH/3T3 cells, selecting for G418-resistant cells. This cell line expressed 'wild type' receptors at a similar level (2.9×10^5 receptors/cell; Table I) to that of other cell lines generated with the mutated receptors as determined by a number of assays described below. NIH/3T3 cells, by comparison, have very low levels of endogenous EGF-R (<500/cell).

pERΔPst, pERB2 and pERB3 were transfected into ψ-2 cells to generate cell lines ψΔP-4, ψB2-2 and ψB3-3, respectively. These lines resulted from G418-resistant colonies and were therefore clonal in nature. They were tested for the production of protein by [^{35}S]methionine and [^{35}S]cysteine metabolic labelling. In as much as the proteins contained mutations in or deletion of the kinase domain, they might not be expected to undergo autophosphorylation. ψΔP-4 expressed a protein of 100 kd mol. wt (Figure 3a, lane b), compared to the 170 kd protein produced by ψER-2 (lane a), as predicted from the structure of the deletion mutant. In addition, the 100 kd protein was precipitable with an antiserum (anti-AI) directed predominantly against the external domain of EGF-R, but not by RK-2, which is against a peptide in the cytoplasmic domain (data not shown).

ψB2-2, derived from transfection with pERB2, contained a protein co-migrating with EGF-R of N22 (Figure 3b, lanes a and b). This is as expected since the mutation is an insertion of only four amino acids.

ψB3-2, derived from transfection with pERB3, contained a protein migrating slightly lower than that of ψER-2 (Figure 3c, lanes a and b) using an antiserum, anti-AI, which recognizes predominantly the extracellular domain of EGF-R. To verify that this protein is not terminating prematurely, we used an antiserum raised against a synthetic peptide of the most c-terminal 13 amino acids of the EGF receptor (R.Kris, S.Werlin and J.Schlessinger, unpublished). This antiserum precipitated the ψB3-2 protein (Figure 3, lane e). An explanation for the slightly enhanced mobility may be that there are alterations in the post-translational modifications of the receptor mutant.

The varying levels of expression of EGF receptor in these and other cell lines appears to be random, depending on the copy number and point of insertion of the gene following transfection. It does not correlate with any of the mutations which in any case are all in the coding region and not in the viral sequences required for expression.

Kinase activity

To analyze whether the mutant proteins retained protein−tyrosine kinase activity, the proteins were immunoprecipitated from each cell line and incubated with [γ-^{32}P]ATP. To distinguish between autophosphorylation and exogenous substrate phosphorylation, purified glyceraldehyde phosphate dehydrogenase (GAPDH) was added to the phosphorylation reaction mixture. GAPDH is an efficient substrate for the EGF receptor kinase with a K_m of 0.45 μM and with phosphorylation exclusively on tyrosine residues (N.Reiss, H.Kanety and J.Schlessinger, submitted for publication). The cell lysates were treated with or without EGF followed by precipitation of the receptor with the anti-c-terminal antiserum. It appears that the EGF-R precipitated from N22 cells is autophosphorylated. Moreover, the immunopurified receptor phosphorylated both GAPDH and the heavy chain of immunoglobulin (Figure 4, lane a) and these activities were inducible with EGF (lane b). In contrast, the mutants with linker insertions in cell line ψB2-2 and ψB3-2 had no detectable autophosphorylation or exogenous substrate phosphorylation activity as compared to the control cell line ψ-2 even after long exposure of the audioradiograms (Figure 4, lanes c−h). Even further exposure, however, did reveal a very low level of autophosphorylation of a 170-kd protein in ψ-2 as well as ψB2-2 and ψB3-2 (not shown). This corresponds to roughly 1000−2000 receptors per cell as determined by EGF-binding studies (see below). NIH/3T3 cells had no detectable autophosphorylation activity (data not shown).

In the GAPDH phosphorylation assay a 2 M KCl wash was used during immunoprecipitation to lower the non-specific background. This wash is required to observe GAPDH phosphorylation, however when milder conditions are used such as in Figure 2 (0.15 M NaCl wash) autophosphorylation is also observed for the EGF receptor expressed in the N22 cells but not with ψB2-2, ψB3-2 or the control ψ-2 cells.

To demonstrate that the expression of EGF receptor in N22 cells is actually from the transfected gene and not the coincidental activation of the endogenous gene, we utilized a human

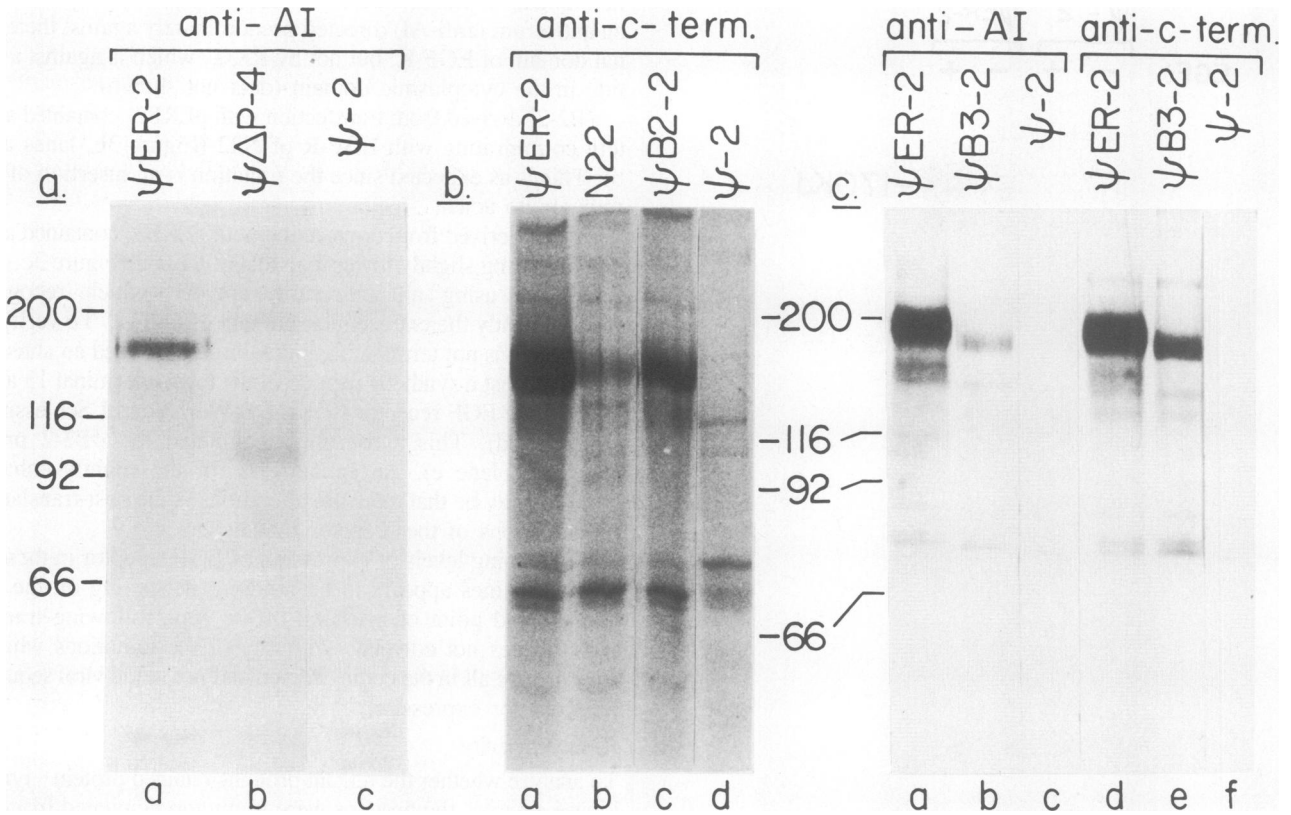

Fig. 3. Metabolic labelling of EGF receptor from transfected cells. (**a**) Subconfluent cells on 10 cm dishes were labelled for 1 h with [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml each) in 2 ml methionine- and cysteine-free DMEM. (**b**) and (**c**) Subconfluent cells on 10 cm dishes were labelled overnight with [^{35}S]methionine and [^{35}S]cysteine (50 μCi/ml each) in 4 ml methionine- and cysteine-free DMEM with 10% fetal calf serum. In each experiment (a, b and c) labelled cells were solubilized, immunoprecipitated with the indicated antisera as described in Experimental procedures and analyzed by 7% SDS-PAGE and fluorography. Anti-AI is directed primarily against extracellular sequences of the EGF receptor while anti-c-terminal serum is directed against a synthetic peptide containing the most c-terminal 13 amino acids of the receptor. Prior to immunoprecipitation cell lysates were normalized for trichloroacetic acid-precipitable radioactivity. The positions of known mol. wt. markers are indicated.

specific monoclonal antibody, 2Eg, provided by B.Defize and S.deLaat. While our anti-c-terminal antiserum precipitated autophosphorylated EGF-receptor from N22 cells and mouse Swiss 3T3 cells, the 2Eg antibody precipitated EGF-R only from N22 cells, demonstrating that the product is coded for by the transfected human clone (Figure 5). The endogenous gene is not significantly expressed in ψΔP-4, ψB2-2 and ψB3-2 cells as shown by the lack of autophosphorylation activity in these lines (Figure 4 and data not shown).

Receptor-v-abl chimera

To determine whether the chimaeric plasmid transformed cells we transfected it into NIH/3T3 cells. We were not able to observe transformed foci due to a high background of spontaneous foci with our NIH/3T3 cells. In addition, the expected transformation efficiency of Abelson-MuLV plasmids is low due to the toxicity of the v-*abl* product (Goff *et al.*, 1982). Therefore, we chose to use Rat-1 cells, an established rat fibroblastic cell line, due to the low background of spontaneous foci with this cell line. The chimaeric plasmid, pRAB, was transfected into Rat-1 cells and transformed foci were observed, albeit at a low efficiency (1–5 foci per 10 μg DNA).

We were able to isolate one focus of cells transformed by pRAB. In order to demonstrate that this line, RRAB-2, was transformed by pRAB and not a spontaneous focus, the cells were tested for autophosphorylation activity. Using EGF-R or v-*abl* specific antisera (Figure 6, lanes c and d) to demonstrate that the phosphorylated protein contains both EGF-R and v-*abl* deter-

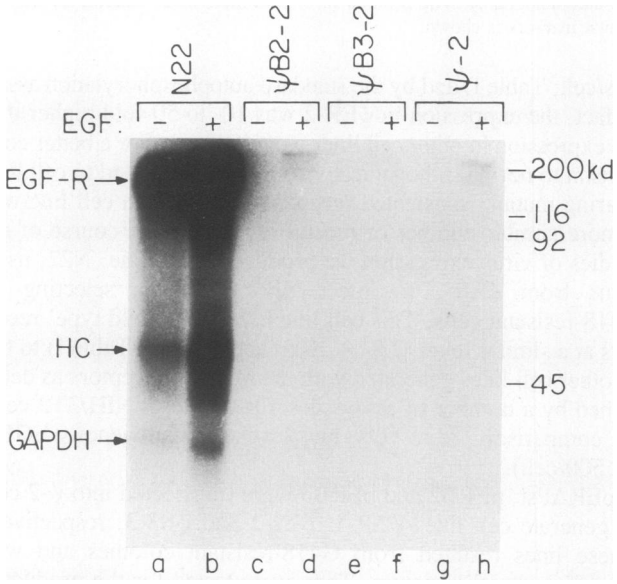

Fig. 4. Autophosphorylation and exogenous substrate phosphorylation of transfected EGF receptor variants. Cell lysates were normalized for amount of protein and treated with or without 5 μg/ml EGF, immunoprecipitated and treated under conditions for autophosphorylation and phosphorylation of the exogenously added substrate glyceraldehyde phosphate dehydrogenase (GAPDH) as described in Experimental procedures. The samples were analyzed by 10% SDS-PAGE and autoradiogrpahy for 12 h. The positions of know mol. wt markers are shown as well as those of EGF-R, immunoglobulin heavy chain (HC) and GADPH.

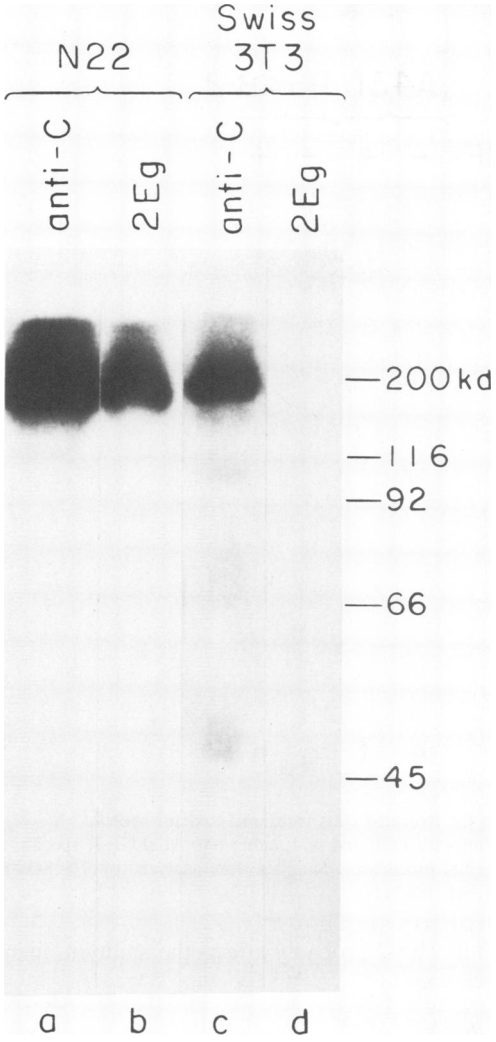

Fig. 5. Immunoprecipitation of EGF receptor with human specific monoclonal antibodies. Equal aliquots of cell lysates normalized for amount of protein were analyzed for autophosphorylation activity as described in Experimental procedures. Lysates from the indicated cells were precipitated with either human specific monoclonal antibody 2Eg (lanes **b** and **d**) or anti-c-terminal serum (lanes **a** and **c**). The autoradiogram is overexposed to reveal possible cross reactivity between the 2Eg antibodies and the murine EGF receptors.

minants. The migration of a polypeptide of apparent mol. wt of roughly 170 000 daltons is consistent with the expected size of the fusion of the external glycosylated domain of 100 000 daltons and the v-*abl* kinase region of 600 amino acid residues. The phosphorylated band co-migrated with receptor from A-431 cells, however in A-431 cells, the EGF receptor is precipitable only with EGF-R-specific antibodies and not with anti-v-*abl* antibodies (Figure 6, lanes a and b). While EGF-R kinase activity is enhanced by EGF (Figure 6, lanes e and f), the activity in the chimaeric protein RRAB-2 was not inducible with EGF (Figure 6, lanes g and h).

EGF binding

The EGF receptor has two affinity classes of EGF binding sites which we will refer to as high and low affinity receptors (Shechter *et al.*, 1978; King and Cuatrecasas, 1982; Rees *et al.*, 1984). These classes are revealed by plotting the binding data by the method of Scatchard (1949). Various concentrations of [^{125}I]EGF were bound to intact cells for 1.5 h at room temperature and cell-associated radioactivity was measured. Non-specific binding was determined by binding [^{125}I]EGF in the presence of 20- to 100-fold excess unlabelled EGF. The binding experiments were performed at room temperature since the high affinity class is temperature dependent and is not apparent at 4°C (King and Cuatrecasas, 1982). Moreover, although 4°C, where internalization is completely inhibited (Hillman and Schlessinger, 1982), would be more optimal, the cells did not remain adhesive to the dish for prolonged incubations at 4°C.

The N22 cells, containing the 'wild type' receptor, clearly displayed both affinity binding classes with a total of 2.9×10^5 receptors per cell (Figure 7a, Table I). Roughly 14% of the binding sites were high affinity while the kd of each class was consistent with previously published data (Table I, Rees *et al.*, 1984). ψ-2, the control cell line used for transfection, had very low levels of EGF binding (Figure 8A). The low level made it difficult to determine by Scatchard plot the number of receptors per cell. Our rough estimate, however, is that ψ-2 cells possess about 1000−2000 endogenous receptors per cell. NIH/3T3 cells had even lower binding, barely detectable above background, <500 receptors per cell. EGF binding studies were performed on each of the transfected cell lines. While the number of receptors varied, presumably according to the efficiency of the transfection, the distribution of high and low affinity sites was drastically altered.

$\psi\Delta$P-4 whose receptors have the cytoplasmic domain deleted, contained only low affinity binding sites though this affinity is similar to the low affinity class of N22 (Figure 7b, Table I). RRAB-2, which also lacks most of the cytoplasmic domain of EGF receptor similarly had only low affinity binding classes (Figure 7e). As RRAB-2 derived from Rat-I cells, we compared its binding to that of the endogenous receptor of Rat-I cells. Though present at low levels, the Rat-I receptor had both affinity sites (Figure 7f). These data demonstrate that cytoplasmic sequences can alter the binding properties of the extracellular EGF binding domain.

The ψB2-2 cell line had both high and low affinity receptors (Figure 7c), thus suggesting that the kinase activity is not the cytoplasmic function required to generate high affinity sites. ψB3-2, however, also containing a kinase-deficient receptor, had only low affinity, but is also altering a region required for the generation of high affinity binding sites.

Receptor internalization

Upon EGF binding, the EGF receptor is internalized rapidly via clathrin-coated pits (reviewed in Schlessinger *et al.*, 1983). We measured the ability of each of the mutants to internalize bound [^{125}I]EGF. EGF was bound for various times at 37°C and cell-associated [^{125}I]EGF determined. To quantitate the amount of EGF receptor internalization, the cells were treated with or without acetic acid for 6 min on ice. This treatment releases any EGF not internalized into cells and thus can be used to determine the rate and extent of receptor internalization (Haigler *et al.*, 1979).

Figure 8a shows the time course of EGF binding to N22 cells. The binding reaches a maximum rapidly but begins to decline after 60 min. The decline was previously demonstrated to be due to degradation of internalized [^{125}I]EGF followed by release of [^{125}I]monoiodotyrosine from the cell (Carpenter and Cohen, 1976). The reduction in total cell-associated ^{125}I radioactivity after 60 min however suggests that in N22 cells the EGF is degraded normally. Moreover, immunoprecipitation experiments after 1 h exposure to EGF utilizing the RK-2 antiserum confirmed that

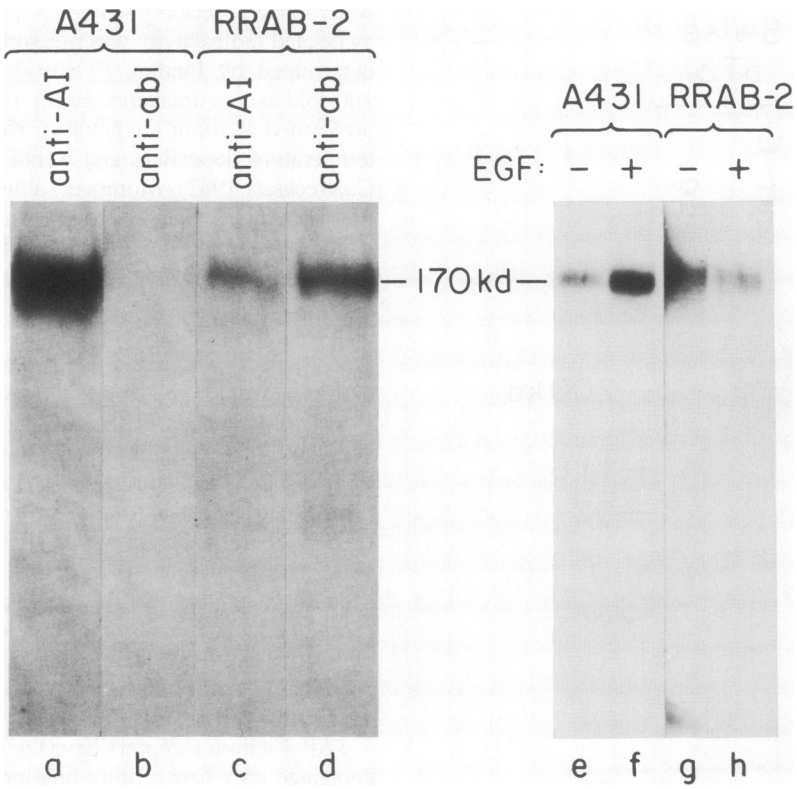

Fig. 6. Immunoprecipitation of EGF-receptor-*abl* chimera. Equal aliquots of cell lysates from the indicated cells were analyzed for specific autophosphorylation activity with (lanes **f** and **h**) or without (lanes **a**−**d**, **e** and **g**) preincubation with EGF and with either anti-AI (EGF-R specific antiserum, lanes **a** and **c**, **e**−**h**) or anti-v-*abl* specific antiserum (lanes **b** and **d**) as described in Experimental procedures. Samples were analyzed by 7% SDS-PAGE and autoradiography. The position of 170 kd mol. wt is indicated as determined from known mol. wt. markers.

the internalized EGF receptor becomes degraded (data not shown).

The extent of internalization of the EGF receptor in N22 cells is shown in Figure 8b as the percent of total cell-associated [^{125}I]-EGF internalized (acetic acid resistant) at each time point. The receptor in N22 cells was internalized rapidly. After 5 min of binding as much as 75% of maximal EGF binding had been obtained but only 20% of it was acetic acid resistant. Within 30 min, however, 75−90% of the bound EGF was internalized. As a control, ψ-2 cells were included in this experiment. The level of binding was so low however to make it impossible to measure the extent of internalization of its endogenous receptor (Figure 8a). NIH/3T3 cells had even lower binding.

A dramatically different pattern was found for $\psi\Delta$P-4 cells whose receptors lack most of the EGF-R cytoplasmic sequences. Maximal EGF binding took longer to achieve (Figure 8a) and the rate and extent of internalization was drastically reduced (Figure 8b). Even after 180 min only 38% of bound EGF was internalized versus 80% for N22 cells at 30 min. RRAB-2 cells, also lacking EGF receptor cytoplasmic sequences, similarly internalized poorly. Moreover, these cells also contain the Rat-1 endogenous EGF receptors (8×10^4 per cell) which internalize rapidly (Figure 8e, f). Taking this into account, it is apparent that the internalization of the chimaeric receptor into Rat-I cells is even poorer than that of $\psi\Delta$P-4. ψB3-2, containing a linker insertion in the cytoplasmic domain and deficient in kinase activity *in vitro*, similarly internalized poorly (Figure 8d). In contrast, ψB2-2, also containing a linker insertion and lacking kinase activity *in vitro*, internalized efficiently (Figure 8d). Thus, kinase activity may not be required for internalization. The insertion of pERB3 in ψB3-2 probably affects the ability to internalize in some other way.

DNA synthesis

The addition of EGF to various cells bearing EGF receptors leads to a mitogenic response (Carpenter and Cohen, 1979). We assayed for this by the incorporation of [^{3}H]thymidine into cells in response to EGF. Although ψ-2 possesses about 1000−2000 endogenous EGF receptors, they do not respond mitogenically to EGF (Figure 9). NIH/3T3 cells also do not respond mitogenically to EGF (data not shown). N22, however, incorporated 5-fold more [^{3}H]thymidine after 18 h incubation with EGF (Figure 9).

The cell lines $\psi\Delta$P-4 and ψB3-2 harboring mutant EGF receptors, defective for high affinity binding, internalization and kinase activity, did not respond to EGF by incorporating [^{3}H]thymidine. Interestingly, the basal level of [^{3}H]thymidine incorporation without EGF varied, but none of these cell lines had a significant increase in response to EGF (Figure 9). The reason for the variability of the basal [^{3}H]thymidine incorporation of the various cells is unclear. Surprisingly, ψB2-2 clearly incorporated [^{3}H]-thymidine in a manner similar to N22 (Figure 9). This stimulation was reproducible in three separate experiments. In addition, the cells were retested for autophosphorylation activity to rule out reversion and had no activity. Titration of the amount of EGF required to stimulate DNA synthesis showed that as little as 1 ng/ml EGF was sufficient to stimulate [^{3}H]thymidine incorporation in both N22 and ψB2-2. Though 'wild type' for binding and internalization, ψB2-2 lacks detectable tyrosine kinase activity *in vitro*, but responds mitogenically to EGF.

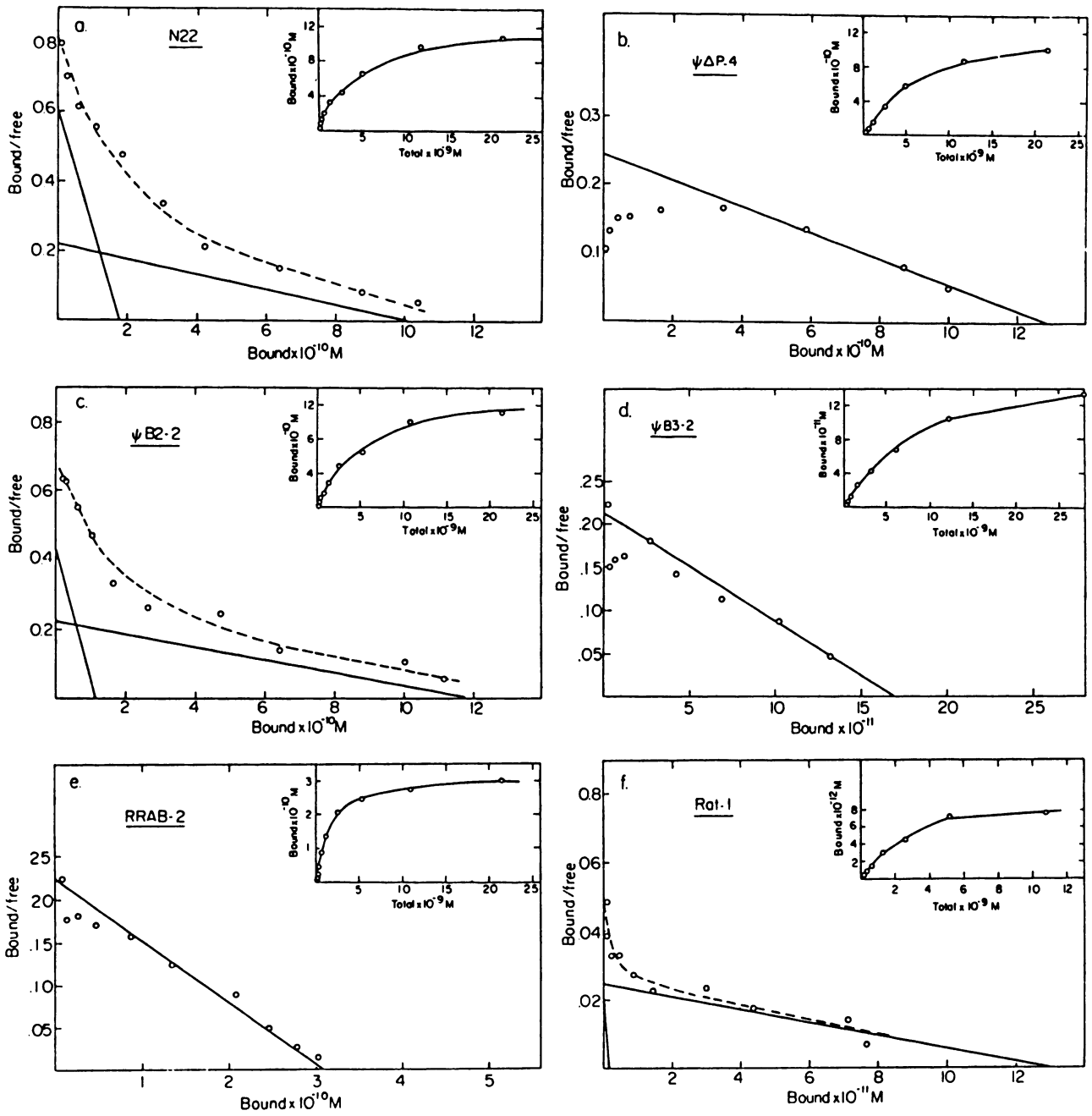

Fig. 7. [^{125}I]EGF binding. EGF binding for each cell line indicated was determined as described in Experimental procedures. Scatchard plots are shown while the inserts show the direct binding data. Each point represents duplicate samples and the binding curves were conistent with three separate determinations. Where two affinity classes of binding sites were found, the dashed line represents the least squares analysis best fit of the data (using a program developed by Stephen Felder). The solid lines represent each affinity class which separately accounts for the total binding. Where one affinity class was found, the solid line represents the best fit for the data assuming one class. For ψΔP-4, the lowest five points were not used for determining the best fit as they may be artificially low due to high background to signal ratios at low binding values.

ψER-2, containing a high level of EGF-R (5.7×10^6 per cell; Table I), had a high basal level of [^{3}H]thymidine incorporation and responded to EGF with a decrease, 20–50%, in [^{3}H]-thymidine incorporation (data not shown). In A-431 cells, which also have an elevated level of EGF receptors, a growth inhibitory effect of EGF has also been reported (Lifshitz *et al.*, 1983).

Discussion

We have made mutations within the cytoplasmic domain of the EGF receptor which affect the binding properties of the extracellular EGF binding domain. In addition, these mutants internalize poorly into the cell in response to EGF binding. An EGF-receptor mutant with undetectable protein–tyrosine kinase activity *in vitro* is still able to bind and internalize efficiently. Surprisingly, cells with this kinase-defective receptor are still able to respond mitogenically to EGF.

EGF binding

The EGF receptor is present on the surface of cells in two forms with different affinities for EGF – on average a high affinity of $K_D = 2 \times {}^{-}10^{10}$ M and a low affinity of $K_D =$

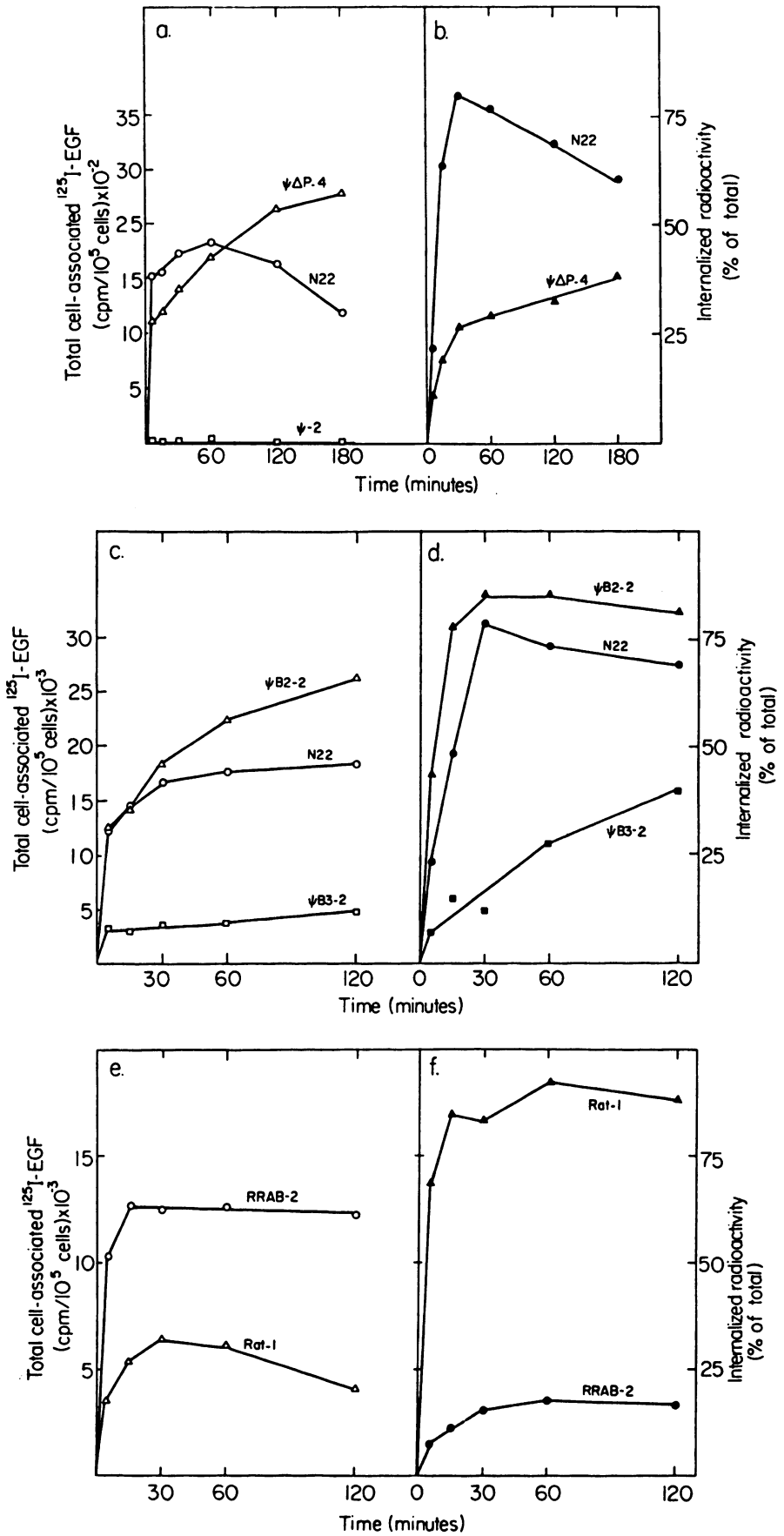

Fig. 8. Internalization of EGF receptor variants. The time course of total cell-associated [^{125}I]EGF binding (open symbols; a,c,e) and [^{125}I]EGF internalized into the cell (closed symbols; b,d,g) was determined for the cell lines indicated as described in Experimental procedures. Internalization by the method of acetic acid sensitivity is expressed as the percent of total cell-associated [^{125}I]EGF internalized at each time point to render the data of different cell lines directly comparable.

Table I. EGF binding: receptor number and affinity. The values for receptor number per cell and binding affinity derived from Scatchard plots and least squares best fit analyses of data in Figure 6 are shown. Either one or two classes of binding sites are shown as appropriate

Cell line	Receptor number/cell	Dissociation constants kd (M)
N22	4.1×10^4	2.8×10^{-10}
	2.5×10^5	4.7×10^{-9}
ψ Δ P-4	2.6×10^5	5.3×10^{-9}
ψ B2-2	3.4×10^4	2.6×10^{-10}
	3.8×10^5	5.2×10^{-9}
ψ B3-2	8.4×10^4	8.2×10^{-9}
RRAB-2	1.8×10^5	1.4×10^{-9}
ψ ER-2	1.0×10^5	3.7×10^{-10}
	5.6×10^6	1.7×10^{-8}
Rat-I	1.1×10^3	9.5×10^{-11}
	7.8×10^4	5.3×10^{-9}

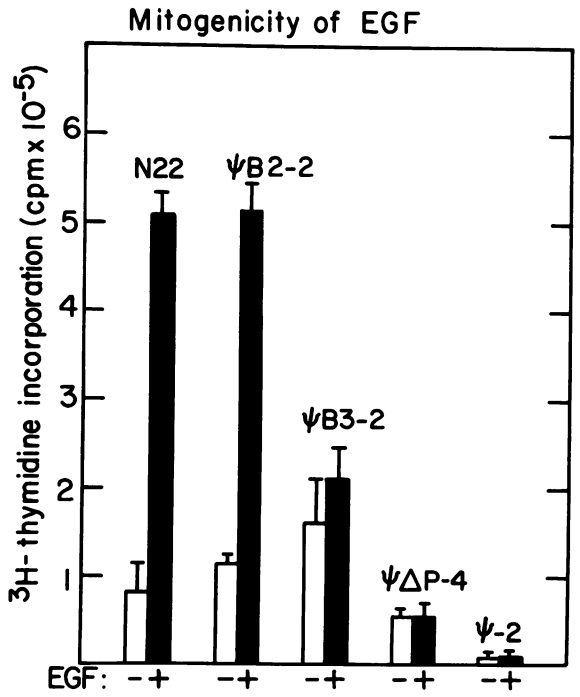

Fig. 9. Mitogenicity of EGF. EGF-induced [^{3}H]thymidine incorporation was determined as described in Experimental procedures. The indicated confluent, starved cells were treated with (shaded bars) or without (open bars) 50 ng/ml EGF 18 h prior to [^{3}H]thymidine addition. [^{3}H]Thymidine was added for 4 h and trichloroacetic acid-precipitable radioactivity was determined. The values indicated are the mean of triplicates and the error bars indicate the standard deviation.

5×10^{-9} M. The difference between these two forms is unknown. The reconstitution of both forms on the cell surface after transfection with EGF-R cDNA demonstrates that one gene codes for both forms.

We made mutations in the cytoplasmic portion of the receptor to determine whether internal interactions and activities of the receptor might be responsible for these two forms. Indeed, deletion of all but 16 amino acids of the cytoplasmic sequences, in pERΔ Pst, resulted in a receptor exhibiting only low affinity binding sites. In addition, a chimaeric gene of the EGF receptor, from the N-terminus up to 44 amino acids on the cytoplasmic side of the membrane, fused to the v-*abl* kinase domain also displayed only low affinity sites. A cytoplasmic function or structure is thus required to have high affinity binding sites. This may not be the kinase activity *per se* since a mutant, pERB2, with a four amino acid insertion in the kinase domain (at amino acid 708) and with undetectable kinase activity *in vitro* exhibits both high and low affinity receptors. Another insertion mutant, pERB3 also within the kinase domain (at amino acid 888) and lacking kinase activity *in vitro*, exhibits only low affinity sites. This insertion may disrupt a structure or other activity required for obtaining high affinity sites. The mutated protein, though entirely intact except for the four amino acid insertion, exhibits a slightly faster mobility on SDS-polyacrylamide gel electrophoresis. This may be due to differences in post-translational modification. If the latter were the case, it may be these modifications which are required to have high affinity sites rather than the specific sequence or structure interrupted by the insertion mutation. The reason for the difference in moblity will be investigated. In addition, further deletions and mutations are being made in the laboratory to more clearly define the sequences required.

A loss of high affinity receptor sites has been observed previously after treatment of cells with the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (Brown *et al.*, 1979; Shoyab *et al.*, 1979; Magun *et al.*, 1980; King and Cuatrecasas, 1982). TPA is able to activate the calcium- and phospholipid-dependent protein kinase C (Castanaga *et al.*, 1982). This enzyme in turn can phosphorylate the EGF receptor, predominantly on a threonine residue (amino acid number 654) which resides 10 amino acids on the cytoplasmic side of the membrane (Hunter *et al.*, 1984; Davis and Czech, 1985). This suggests that phosphorylation of threonine-654 results in a loss of high affinity sites. However, it is clear that TPA causes phosphorylation of additional sites of the EGF receptor which could also play a role in the regulation of the affinity of the receptor for EGF (Davis and Czech, 1985). Indeed phosphorylation of threonine-654 does not appear to be sufficient to explain the loss of high affinity sites in our mutants. We have analyzed a point mutant at threonine-654 which suggest that factors in addition to phosphorylation of Thr654 may be responsible for the effects of TPA on the affinity of EGF-receptor (J.Schlessinger, A.Ullrich, T.Dull, R.Prywes and E.Linveh, in preparation). Thus, the mechanisms of control of high and low affinity forms appear to be more complex.

Yarden and Schlessinger (submitted) have recently described an allosteric aggregation model for the activation of the EGF-receptor kinase. Moreover, evidence was provided that the receptor exists in an equilibrium between monomeric and dimeric forms. EGF induces dimers to form and it is proposed that these dimers would have higher affinity for EGF, with EGF binding stabilizing that form. In addition, EGF binding and dimerization result in activation of the kinase activity. Using this as a working model it is possible to explain the phenotype of the mutants in terms of their ability to dimerize. Cytoplasmic sequences deleted in pERΔ Pst and pRAB, or interrupted in pERB3 would therefore be required for the efficient formation of dimers. In addition, protein kinase-C phosphorylation might act by shifting the equilibrium toward monomers. Work is in progress to determine the aggregation state of the mutant proteins and the affect of phosphorylation by kinase-C on their aggregation state.

Internalization

The EGF receptor internalizes via clathrin-coated pits upon EGF binding (reviewed in Carpenter and Cohen, 1979; Schlessinger

et al., 1983). This route of internalization is specific and excludes many surface proteins. The receptors for polymeric IgA/IgM, asialoglycoproteins, transferrin, insulin and low density lipoproteins (LDL) among others also all enter the cell via clathrin-coated pits (reviewed in Brown *et al.*, 1983). The mechanism of localization within coated pits is not known, however Lehrman *et al.* (1985) have demonstrated that mutations within the cytoplasmic domain of LDL receptor strongly reduce the ability of receptors to internalize. We can now confirm these results in the EGF receptor system where deletion of cytoplasmic sequences also lowers the ability of the EGF receptor to internalize.

There are several important differences between the LDL and EGF receptors. The LDL receptor has only 50 amino acids in its cytoplasmic domain and no known enzymatic activity while the EGF receptor has 542 amino acids encoding a tyrosine kinase activity and specific autophosphorylation sites. In addition, the EGF receptor requires EGF to internalize and is not recycled while the LDL receptor is constitutively internalized with or without LDL and can be recycled to the cell surface (Brown *et al.*, 1983). Therefore, in the EGF receptor system there are at least two questions of interest with regard to internalization: which domains are essential for EGF binding to transmit the signal for the receptor to internalize and which domains are required for internalization specifically into clathrin-coated pits? At present we cannot distinguish between these sequences, but can conclude that cytoplasmic sequences are required for at least one of these functions since a deletion of all but 16 amino acids of the cytoplasmic domain, as well as other mutations introduced into this region, lead to endocytosis-defective receptor mutants.

The protein−tyrosine kinase activity, however, may not be required for internalization since a linker insertion mutant at amino acid 708 (pERB2), with defective kinase activity *in vitro*, internalizes like 'wild type' receptor. Another linker insertion mutant at amino acid 888 (pERB3) also lacks tyrosine kinase activity *in vitro*, but does not internalize efficiently. This mutant, which also lacks high affinity binding sites, may provide a clue as to which specific structures are required for efficient internalization. More mutants must be made to define precisely these sequences.

The internalization mtuants we have described do internalize partially. The initial kinetics are faster than EGF receptor turnover without EGF ($t_{1/2}$ = 10 h in human fibroblasts; Stoscheck and Carpenter, 1984). It will be of interest to determine whether this internalization is via coated pits or utilizes another mechanism.

Mitogenicity

The transfected EGF receptor gene was able to reconstitute the ability of NIH/3T3 cells to respond mitogenically to EGF. Increased DNA synthesis was measured by the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material after incubation of cells with or without EGF. Surprisingly, the cell line ψB2-2, containing a kinase-defective protein, showed an EGF-stimulated incorporation of [^{3}H]thymidine, i.e., responded mitogenically to EGF. ψB2-2 is 'wild-type' for EGF binding and internalization. The deletion mutant, in $\psi\Delta$P-4, and insertion mutant, in ψB3-2, are deficient in these activities as well as tyrosine kinase activity and do not respond mitogenically to EGF. Finally, neither of the control cell lines ψ-2 nor NIH/3T3 cells respond mitogenically to EGF.

We hesitate to suggest that the positive result with ψB2-2 infers that kinase activity is not required for the mitogenic signal and that some other unknown function is necessary. There is an extensive literature supporting the importance of the tyrosine-specific kinase. There is conservation of this domain and activity through a large number of growth factor receptors and oncogene products (reviewed in Bishop, 1983 and Hunter and Cooper, 1985). In addition, in several oncogenic retroviruses containing these related oncogenes, temperature-sensitive or transformation-defective mutants have been found that are temperature sensitive or defective in protein−tyrosine kinase activity (reviewed in Bishop, 1983; Parsons *et al.*, 1984; Stone *et al.*, 1984; Prywes *et al.*, 1985).

Another possible explanation is that while defective in our *in vitro* assays for autophosphorylation and exogenous substrate phosphorylation, the mutated protein is still active in the intact cells. Namely, the insertional mutation at the kinase domain renders the immunoprecipitated enzyme unstable, while in intact cells this receptor mutant possesses kinase activity capable of phosphorylating the physiologically important substrates. It would be of interest to assay the *in vivo* kinase activity of the receptor, however, this is complicated by the low phosphotyrosine level in the cells and the unknown nature of physiologically important substrates. While an EGF-induced increase in phosphotyrosine levels can be observed in A431 cells which overexpress the EGF receptors (Hunter and Cooper, 1981) it is difficult to observe a change in cells with lower EGF-R expression such as human foreskin fibroblasts (1×10^5 receptors/cell; Yarden, 1985), N22 cells and the cells expressing the various EGF-receptor mutants. Further experiments are currently being performed in order to resolve this question.

Receptor-v-abl chimaera

The chimaera between the EGF binding and transmembrane domains of EGF receptor and the v-*abl* kinase domain of Abelson murine leukemia virus (A-MuLV) was able to morphologically transform Rat-I fibroblast cells. The chimaeric protein is precipitable with both EGF-R and v-*abl* specific antisera, has autophosphorylation activity and binds EGF. EGF, however, cannot activate the heterologous kinase activity nor can it induce the endocytosis of the chimaeric molecule.

The v-*alb* kinase domain alone, expressed in a retrovirus construct, is not sufficient to transform NIH/3T3 fibroblasts (Prywes, 1984) nor Rat-1 cells (Peter Jackson, personal communication). This appears to be due to the requirement for the viral structural (gag) sequences normally fused to the N-terminus of v-*abl* in A-MuLV. These sequences provide a site for covalent attachment of the fatty acid myristrate to the amino terminal amino acid glycine. Myristylation of an amino terminal glycine is present in pp60^{src}, the transforming protein of Rous sarcoma virus (Buss and Sefton, 1985). Mutants that do not myristylate, while retaining tyrosine kinase activity, do not transform fibroblasts. In addition, the mutant proteins are no longer membrane associated as is the 'wild type' protein (Cross *et al.*, 1984; Pellman *et al.*, 1985). Thus, by analogy, the EGF receptor sequence appears to activate the transforming potential of the v-*abl* kinase by localizing the kinase to the inner membrane surface. It will be interesting to learn whether the *bcr* gene fused to c-*abl* in chronic myelogenous leukemia cells (Groffen *et al.*, 1984; Shtivelman *et al.*, 1985) is also activating c-*abl* by localizing it to the membrane surface. It is noteworthy that EGF is unable to enhance the tyrosine kinase activity and internalization of the chimaeric molecule. Hence, the tailoring of a kinase domain to ligand binding region does not necessarily maintain the regulatory properties of a 'wild type' receptor. Still, it is possible that with other chimaeric constructs, the ligand will be able to stimulate the cytoplasmic *abl*-kinase function.

Materials and methods

Plasmid constructions

The EGF receptor cDNA was cloned into the retrovirus expression vector in several steps. First the full length cDNA was pieced together from overlapping λ phage cDNA clones λHER-A64 and λHER-A21 (Ullrich *et al.*, 1984) and subcloned into pBR322. The final subclone extended from the *Sst*I site, 20 bp before the initiation codon for translation (nucleotide no. 168, numbering according to Ullrich *et al.*, 1984) to an *Xmn*I site 150 bp after the termination codon (nucleotide no. 3970). Synthetic *Xho*I linker DNA was used to convert both ends to *Xho*I sites. In addition the *Pvu*II site of pBR322 was changed to an *Xho*I site in the same way and used for subcloning. The EGF receptor cDNA fragment with *Xho*I ends was then placed into the expression plasmid. pZIP-NeoSV(X)I (Cepko *et al.*, 1984) which was slightly altered for this purpose. The *Sal*I site of pBR322 sequence was destroyed and the *Bam*HI site, normally used for inserting cDNAs, was altered to a *Sal*I site using linker DNA. *Xho*I and *Sal*I have compatible cohesive ends such that the EGF-R fragment described above could be ligated into this new *Sal*I site. This final plasmid in the correct orientation is pER, diagrammed in Figure 1a.

pERΔPst was constructed by partial digestion of pER with *Pst*I, purification by agarose gel electrophoresis of the desired sized band and religation of the DNA. This resulted in deletion of nucletoides 2238−3581. The coding sequence goes out of frame at the deletion junction.

pERB2 and pERB3 were generated by partial digestion of pER with restriction endonuclease *Bam*HI. Single-cut DNA was purified by agarose gel electrophoresis, the staggered ends filled in with Klenow fragment of DNA polymerase I and 8 bp *Sal*I synthetic linker DNA was ligated on. Excess linker DNA was removed by digestion with *Sal*I and agarose gel electrophoresis. The resulting DNA was ligated to close the circle. This process results in the insertion of 12 bp, four from the filled-in *Bam*HI site and eight from the linker, leaving the coding region in frame. pERB2 has a *Sal*I linker inserted at the *Bam*HI site at nucleotide no. 2378, pERB3 at nucleotide 2917.

pRAB fused the EGF-R gene to a section of v-*abl*. Sequences of EGF-R up to the *Eco*RI site at nucleotide 2317 were fused to a *Hinc*II to *Xho*I fragment of pAB160 (Prywes *et al.*, 1983), containing the Abelson murine leukemia virus genome. A 12 bp *Eco*RI synthetic DNA linker was ligated onto the *Hinc*II site to facilitate the fusion and to retain the coding region in frame. This construct utilized pER for EGF-R sequences and resulted in the same overall vector structure.

Each of the plasmids constructed were tested for the presence of the desired mutation by digestion with appropriate restriction endonucleases. The methods for plasmid constructions were performed as described (Prywes *et al.*, 1985) or by other standard procedures (Maniatis *et al.*, 1982). Enzymes were provided by New England Biolabs and Boehringer Mannheim and were used as described by the manufacturers. Synthetic *Sal*I DNA linkers were provided by New England Biolabs.

Cells and transfections

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. Transfections were by the calcium phosphate precipitate technique as described (Wigler *et al.*, 1979) and modified (Prywes *et al.*, 1983). Ten micrograms of cloned DNA was used to transfect one 10 cm plate of cells seeded with 5×10^5 cells 24 h prior to transfection. Two to three days later the cells were split 1:20 and put under selection with the antibiotic Geneticin G418, 0.8 mg/ml (GIBCO).

Labelling, immunoprecipitation and autophosphorylation

Cells were labelled with [^{35}S]methionine and [^{35}S]cysteine (Amersham) at 50 μCi/ml and 25 μCi/ml, respectively, in methionine- and cysteine-free DMEM. Fetal calf serum (10%) was added for overnight labeling. Solubilization, immunoprecipitation and phosphorylation (independent of metabolic labeling) has been described (Kris *et al.*, 1985). For exogenous substrate phosphorylation of glyceraldehyde phosphate dehydrogenase (GAPDH) the following modifications were made. Cell lysates were normalized for amount of protein as determined by the method of Bradford (1976). 50 mM β-mercaptoethanol and 50 mM EDTA was added to the solubilization buffer (20 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1.0 mM EGTA; 0.01 mg/ml aprotinin; 1 mM PMSF). The immunoprecipitates on protein A-sepharase (Pharmacia) were washed twice with 2 M KCl in HNTG (20 mM Hepes, pH 7.5; 150 mM NaCl. 0.1% Triton X-100; 10% glycerol) followed by two washes with phosphorylation buffer (50 mM Hepes, ph 7.5; 150 mM KCl; 0.1% NP40; 0.1% Triton X-100; 10% glycerol) followed by two washes with phosphorylation buffer (50 mM Hepes, pH 7.5; 150 mM KCl; 0.1% NP40; 5 mM $MnCl_2$). The phosphorylation was with [γ-^{32}P]ATP, 1 μCi per assay (Amersham) and 2 μM GAPDH (Sigma) was carried out for 20 min at room temperature. The reaction was terminated with 3 × sample buffer, boiled for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis in the standard fashion.

Antisera were as follow: RK-2 (Kris *et al.*, 1985) is a rabbit antipeptide antiserum. Anti-c-term is a rabbit serum raised against the c-terminal 13 amino acid peptide of human EGF-R (R.Kris, S.Werlin and J.Schlessinger, unpublished). Anti-AI is a rabbit serum prepared against A-431 cell membranes and had reactivity predominantly against extracellular sequences of the EGF receptor. Anti-v-*abl* is a rabbit serum generated against a peptide of the kinase domain of v-*abl* (a gift of N.Taylor). The human EGF-R specific monoclonal antibody, 2Eg, was a gift of B.Defize and S.deLaat.

EGF binding

[^{125}I]EGF was prepared by the lactoperoxidase method (Marchalonis, 1969) using Na^{125}I from Amersham and mouse EGF from I.D.L., Jerusalem. For binding studies cells were seeded at 1×10^5 cells per well 2 days previously in 24-well costar dishes. These wells were pre-coated with 15 μg/ml fibronectin (Bioprocessing, Ltd) in phosphate buffered saline (PBS) for 30 min at room temperature to ensure adhesion of cells. Prior to binding each well was washed 2 × with PBs and then incubated for 1.5 h with various concentrations of [^{125}I]-EGF at room temperature. Wells were then washed 2 × with ice-cold PBS and solubilized with 1 ml of 0.2 N NaOH. After 30 min at 37°C the mixture was transferred to tubes for couunting of total cell-associated radioactivity. Non-specific binding was determined by parallel incubations of each concentration of [^{125}I]-EGF with 200 ng/ml cold EGF or 30 × the concentration of [^{125}I]EGF, whichever was higher. All incubations were performed in duplicate. Specific binding was calculated as total minus non-specific cell-associated radioactivity.

The number of receptors and K_D for binding was determined from the Scatchard plot using a least squares analysis program (developed by Stephen Felder) to find a best fit for the data, assuming either one or two binding classes.

Internalization

The method of acetic acid treatment was used to measure the cell surface versus internalized bound EGF (Haigler *et al.*, 1979). [^{125}I]EGF was bound as described above except that binding was at 37°C for various times. At indicated times, dishes were placed on ice, washed 2 × with cold PBS and treated with or without 0.5 N acetic aicd, pH 2.7; 0.15 M NaCl for 6 min on ice with one change after 5 min. Cells were then solubilized with 0.2 N NaOH and radioactivity determined. [^{125}I]EGF was bound at a concentration of 100 ng/ml. Non-specific binding was determined in parallel with 100 ng/ml [^{125}I]EGF in the presence of 3 mg/ml native EGF. Specific binding with and without acetic acid was determined in duplicate. Non-specific binding with and without acetic acid was determined by single incubations each.

DNA synthesis

Cells were seeded at 1×10^5 cells per well in 24-well Costar dishes pre-coated with fibronectin as described above. The cells were then grown for 2 days, the media changed to DMEM/1% fetal calf serum for 24 h and then incubated with or without EGF at 50 ng/ml for 18 h. [^{3}H]Thymidine, 5 μCi per well, was added for 4 h at 37°C. Cells were washed 2 × with ice-cold PBS, cold 5% trichloroacetic acid was added and left 30 min on ice. Wells were then washed 2 × with PBS, solubilized with 0.2 N NaOH and radioactivity was determined.

Acknowledgements

We gratefully acknowledge Stephen Felder for developing the program for analysis of the binding data and Nachum Reiss for help with the exogenous substrate assay. This work was supported by grant CA-25820 from the National Institute of Health and a grant from the US-Israel Science Foundation.

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Received on 16 May 1986