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Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector

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The mechanism by which the binding of epidermal growth factor (EGF) to specific cell surface receptors induces a range of biological responses remains poorly understood. An important part of the study of signal transduction in this system involves the production of sufficient native and mutant EGF receptor species for X-ray crystallographic and spectroscopic analysis. Baculovirus vectors containing the cDNA encoding the human EGF receptor protein have here been utilized to infect insect cells. This results in expression of a 155-kb transmembrane protein which is recognized by four antibodies against different regions of the human EGF receptor. Studies with tunicamycin, monensen and endoglycosidase H show the difference in size between the recombinant and the native receptor is due to alterations in glycocsylation. Studies of [^{125}I] EGF binding shows a K_d of 2×10^{-9} M in intact infected insect cells which falls to 2×10^{-7} M upon detergent solubilization. The recombinant protein exhibits an EGF-stimulated tyrosine protein kinase activity and an analysis of tryptic peptides shows that the phosphate acceptor sites are similar to those of the EGF receptor isolated from A431 cells. These observations indicate that functional EGF receptor can be expressed in insect cells, and furthermore, this system can be used for large-scale production.

Key words: glycosylation/SF9 cells/structural analysis

Introduction

The human epidermal growth factor (EGF) receptor is a 170-kd transmembrane glycoprotein that mediates the mitogenic response of target cells, from all three germ layers to EGF and EGF-like polypeptides (for recent review, see Carpenter, 1987). The complete amino acid sequence has been deduced from nucleotide sequencing of cDNA clones (Ullrich *et al.*, 1984). Based on this, a structural model for the receptor has been proposed which comprises an extracellular EGF-binding domain, a short hydrophobic transmembrane segment and an intracellular domain which possesses a tyrosine protein kinase activity. The kinase, which is stimulated by binding of EGF to the receptor, can mediate autophosphorylation of several tyrosine residues, three of which are located close to the carboxy terminus (Downward *et al.*, 1984). The kinase can also phosphorylate a variety of other protein and synthetic peptide substrates (Hunter, 1984; Downward *et al.*, 1985).

The binding of EGF to its plasma membrane receptor initiates many diverse responses. These include receptor down-regulation which involves clustering and internalization of receptor-bound EGF followed by receptor recycling or degradation (Carpenter and Cohen, 1976; Schlessinger, 1980), alterations in membrane ion transport (Moolenaar *et al.*, 1982) and changes in cell morphology, related perhaps to cytoskeletal effects (Chinkers *et al.*, 1979, 1981). A mitogenic response to EGF in target cells is obtained only after 6–8 h of continuous exposure to EGF (Carpenter and Cohen, 1976; Schechter *et al.*, 1978).The mechanism by which EGF induces these biological effects remains poorly understood and the relationship, if any, of the early responses to activation of the tyrosine protein kinase and to the subsequent mitogenic response remains to be elucidated. At present the tyrosine protein kinase activity is the only EGF-induced activity of the receptor which has been characterized.

The putative model for the receptor structure suggests that it has a single α-helical transmembrane domain. This model places constraints on mechanisms for signal transduction and has led to two distinct proposals to explain the process (Staros *et al.*, 1985; Yarden and Schlessinger, 1987a,b). The first is an intramolecular model which requires that ligand binding alters the interaction of the external domain with the hydrophobic membrane stretch of the EGF receptor, and that this transfers a conformational change to the cytoplasmic kinase domain. An alternative model involves an intermolecular allosteric process in which ligand-induced receptor oligomerization leads to the activation of the protein kinase domain through interaction between neighbouring cytoplasmic domains, thus by-passing the requirement imposed by the first model for conformational change to be transferred through the single transmembrane region.

An essential part of the study of ligand–receptor interaction involves purification of sufficient native EGF receptor and mutant receptors for spectroscopic analysis by NMR and optical methods, and for full X-ray crystallographic determination of its three-dimensional structure. Attempts to express eucaryotic proteins for such analysis in bacteria have not been entirely successful. Bacterially produced eucaryotic proteins have a poor solubility, and bacterial cells are unable to modify and glycosylate eucaryotic proteins. Large-scale preparation of A431 human vulval carcinoma cells, which have $\sim 2 \times 10^6$ EGF receptor molecules per cell (Fabricant *et al.*, 1977), could provide the required amount of receptor protein for structural analysis of normal receptors. However, to produce mutant receptors in sufficient quantity requires alternative approaches. DNA-mediated gene transfer into cultured animal cells could be employed but this has often resulted in insufficient levels of protein expression (personal observation). For these reasons an alternative expression system known to be capable of inducing high levels of recombinant protein has been investigated here. This system (for review, see Summers and Smith, 1987)

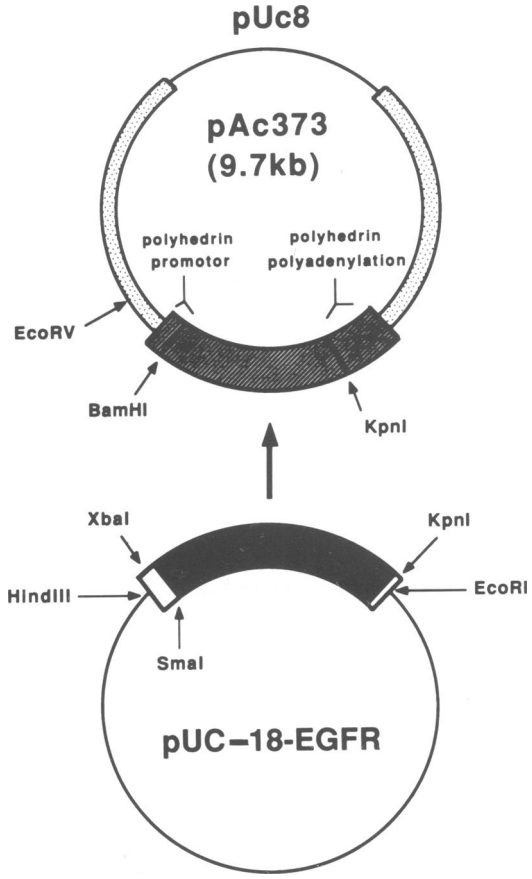

Fig. 1. Assembly of vector pAc373-EGFR. The coding sequence of the polyhedrin protein was removed from the vector pAc373 by a *Bam*HI−*Kpn*I digest, leaving the polyhedrin promoter (−8bp) and polyadenylation sequences intact. The left and right arms of this vector are homologus to the flanking sequences of the polyhedrin gene in the wild-type virus (▨), facilitating recombination when pAc373 is co-transfected with wild-type virus DNA. The cDNA encoding the full length human EGF receptor (▬), was removed from pUC18-EGFR by an *Xba*I−*Kpn*I digest and transferred to the modified pAc373. The resulting construct pAc373-EGFR contained all but eight nucleotides of the polyhedrin 5′-untranslated leader sequence fused to 20 nucleotides from the polylinker of pUC18 (▭), in continuation with 17 nucleotides of the receptor 5′-non-coding sequence.

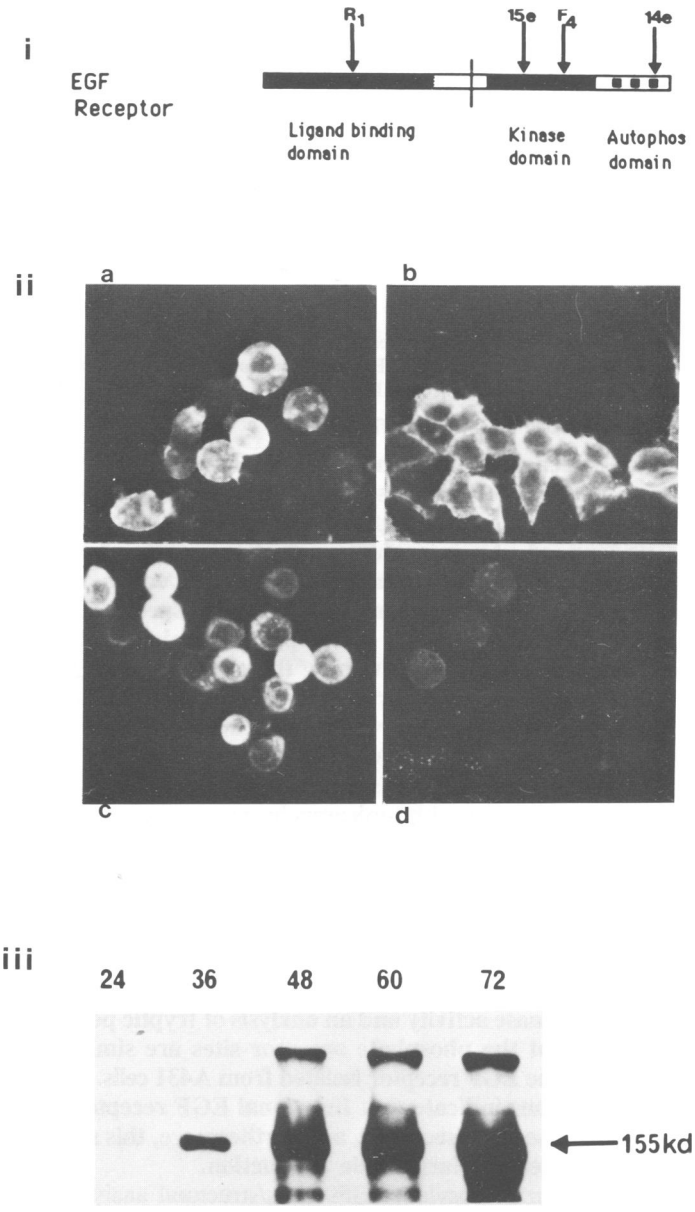

Fig. 2. Characterization of the recombinant EGF receptor using antibodies. **(i)** A schematic representation of the domain structure of the EGF receptor and the epitopes recognised by the different antibodies used in this study. Antibody R_1 recognizes epitopes on the external domain, Antisera 15E, F4 and 14E were raised against peptides corresponding to the amino acid residues (736−747 ATP binding site), (1005−1016) and (1184−1196 phosphorylation site 1) respectively (Gullick *et al.*, 1985). **(ii)** Intact SF9 **(a)** and A431 **(b)** were treated with Mab R_1 and binding detected by fluorescein-conjugated goat anti-mouse IgG. Triton-permeabilized insect cells (**c** and **d**) were treated with Mab F4 (c) or with Mab F4 plus competing peptide 2E (d), antibody binding was detected as with Mab R_1. **(iii)** 10^5 insect cells were lysed, 24, 36, 48,60 and 72 h post-infection, and the lysates immunoblotted with anti-15E antiserum.

utilizes cell lines from the Fall Army worm *Spodoptera frugiperda* (SF9) which can be infected by the naturally occurring baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). During lytic infection a late gene product for the baculovirus (polyhedrin) is expressed and can account for up to 50% of total cellular proteins (Summers and Smith, 1985). The polyhedrin gene, however, is not essential for production of infectious extracellular virus. Therefore expression vectors that use the strong AcNPV polyhedrin promoter to drive foreign genes have been developed (Smith *et al.*, 1983). By using this approach, large amounts of eucaryotic proteins have been successfully produced, including human fibroblast interferon (Smith *et al.*, 1983), human c-*myc* (Miyamoto *et al.*, 1985) and human interleukin 2 (Smith *et al.*, 1985). In addition, insect cells can perform many of the post-translational modifications carried out by higher eucaryotes including accurate signal peptide cleavage (Smith *et al.*, 1985), phosphorylation (Miyamoto *et al.*, 1985), glycosylation (Smith *et al.*, 1983) and the ability to induce extracellular secretion of proteins (Smith *et al.*, 1985). These features of the baculovirus system make it an attractive proposition for the large-scale production of normal and mutant EGF receptors for biophysical study. As a preliminary to large-scale production, we have characterized the human EGF receptor expressed in SF9 cells infected with a recombinant baculovirus containing the

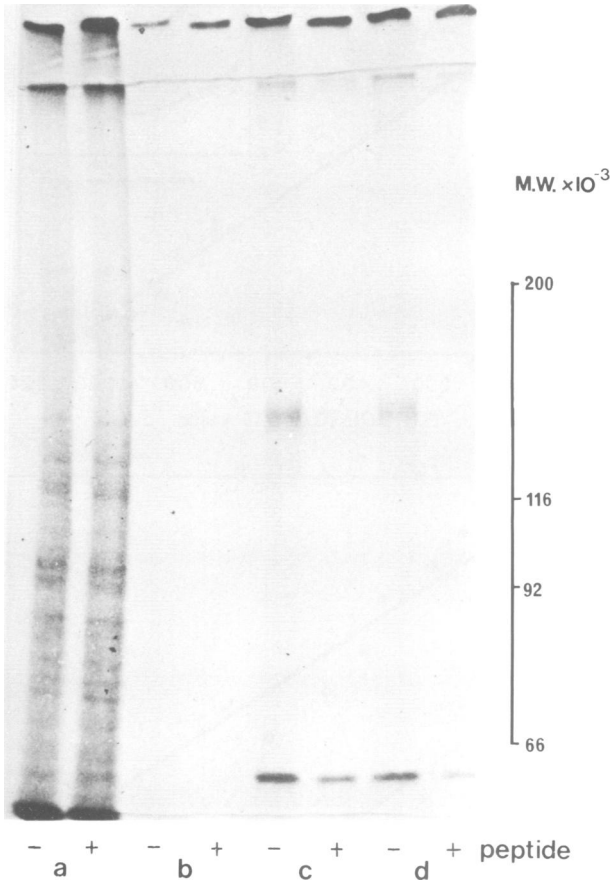

Fig. 3. Immunoprecipitation of [^{35}S]methionine-labelled insect cells. Uninfected (**a**), wild-type infected (**b**), and recombinant virus-infected (**c** and **d**) insect cells were lysed 48 h post-infection. The lysates were immunoprecipitated with anti-15E antiserum (a,b,c) or with anti-14E antiserum (d) with (+) or without (−) competing peptide.

cDNA encoding for the human EGF receptor under the transcriptional control of the viral polyhedrin promoter. The expressed protein has the structural and functional characteristics of the native EGF receptor found on A431 cells. It therefore seems appropriate to use this baculovirus system for future large-scale production.

Results

The strategy used for the assembly of the insect vector containing the EGF receptor cDNA is outlined in Figure 1. The plasmid pAc373-EGFR contains all but the first 8 bp of the polyhedrin 5′-untranslated region, followed by 20 nucleotides from the polylinker of pUC-18 together with 17 nucleotides of the 5′-non-coding sequence. To transfer the EGF receptor cDNA into the vAcNPV baculoviral genome, SF9 insect cells were co-transfected with pAc373-EGFR and wild-type AcNPV DNA. Recombinant virus was initially identified by DNA hybridization analysis of infected cells and later, visually, by selecting for occulsion negative plaques, i.e. those lacking the polyhedrin protein.

The properties of the recombinant receptor produced using the baculovirus expression system were first analysed by structural criteria based upon the use of antibodies known to recognize epitopes in the human receptor and second by

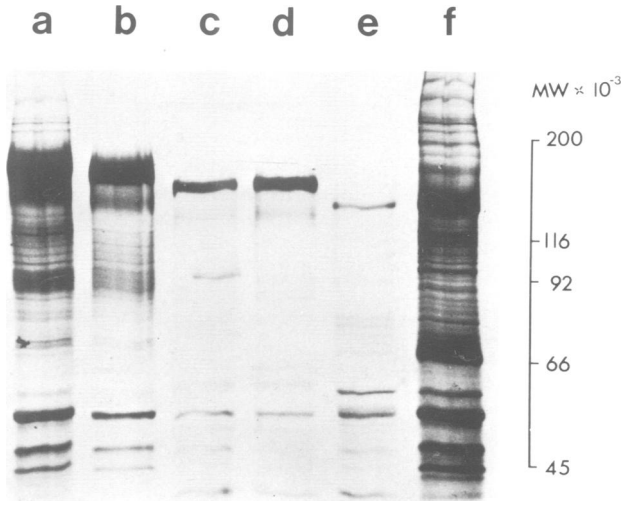

Fig. 4. Immunoprecipitation with Mab R_1 of [^{35}S]methionine-labelled insect and A431 cells treated with tunicamycin or monensin. (**a**) Untreated A431 cells, (**b**) A431 cells treated with monensin (**c**) untreated infected SF9 cells (**d**) infected SF9 cells treated with monensin (**e**) infected SF9 cells treated with tunicamycin (**f**) A431 cells treated with tunicamycin. As viral infection inhibits cellular protein synthesis, the majority of [^{35}S]methionine-labelled proteins are of viral origin. To obtain the correct exposure for track c,d and e, tracks a,b and f were overexposed.

ligand binding and ligand-stimulable tyrosine protein kinase activity.

Receptor expression

Expression of the human EGF receptor by infected SF9 insect cells was examined using antibodies raised against the human EGF receptor or various synthetic peptides, corresponding to different regions of the receptor molecule [Gullick *et al.*, 1985, 1986; Waterfield *et al.*, 1982; see Figure 2(i)]. Human A431 carcinoma cells which overexpressed the EGF receptor (Fabricant *et al.*, 1977) were used as controls. Immunofluorescent staining of formaldehyde-fixed SF9 insect and A431 human cells before and after permeabilization by brief treatment with Triton X-100 was carried out. The results of this analysis with the monoclonal antibody F4 (Mab F4) which recognises the internal kinase domain and Mab R_1 which recognises the external domain of the EGF receptor are shown in Figure 2(ii). Examination of infected intact insect or A431 cells using Mab R_1 revealed intense plasma membrane fluorescence. Antibodies to the receptor's internal domain (Mab F4) did not react with intact cells unless the cells were permeabilised with detergent, when strongly positive staining was obtained. The Mab F4 staining was specific since it could be blocked by pre-incubation of the antibody with the immunizing peptide (2E). These results strongly suggest that in infected SF9 insect cells the EGF receptor is correctly orientated with respect to the plasma membrane. The production of receptor protein was monitored during the post-infection period. The intensity of fluorescence and the number of cells showing fluorescence increased with time from 36 h post-infection to peak at ~ 72 h at which time viral lysis of cells was occurring. Western blot analysis confirmed the increase in EGF receptor protein production with time after infection [Figure 2(iii)]. To avoid problems

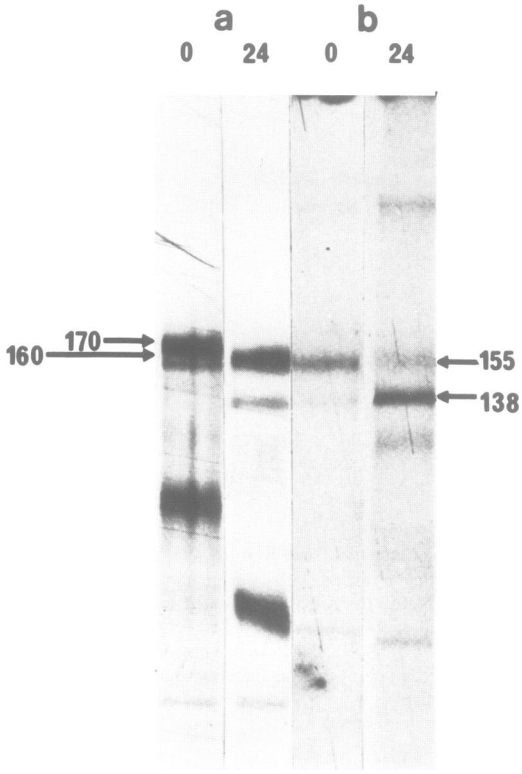

Fig. 5. Endoglycosidase H digestion of the immunoprecipitated receptor. Immunoprecipitates of A431 and SF9 cell lysates were treated for 24 h with 5 mU of Endoglycosidase H. (**a**) A431-derived EGF receptor after 0 and 24 h digestion. (**b**) SF9-derived EGF receptor after 0 and 24 h of digestion. The numbers in the left and right margins indicate the molecular sizes $\times 10^{-3}$

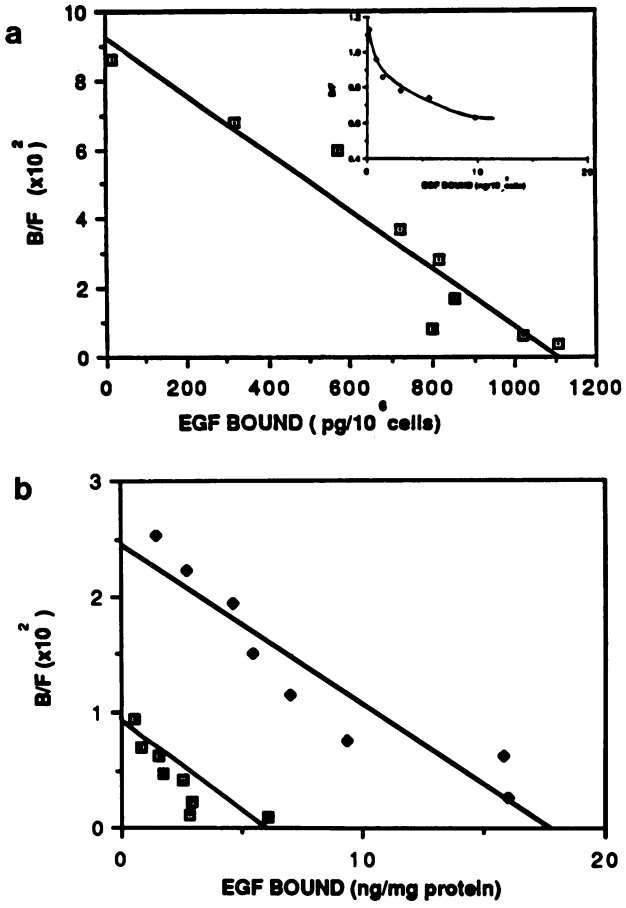

Fig. 6. Receptor–ligand binding. [^{125}I]EGF and increasing concentrations of unlabelled EGF were used to generate Scatchard plots of receptor ligand binding for intact A431 cells. **a. insert** ●; intact insect cells, **a** □; solubilized A431 cells, **b** ◆; and solubilized SF9 cells, **b** □.

associated with cell lysis, cells were harvested 48 h post-infection.

To further characterize the EGF receptor produced in this insect cells system ($_i$EGFR), lysates of [^{35}S]methionine-labelled infected SF9 cells were immunoprecipitated with, anti-14E and anti-15E antisera [see Figure 2(i)]. The results (Figure 3) show that a protein is expressed which has the conformationally dependent determinant recognized by Mab R_1 together with the determinants recognized by antisera to the main *in vivo* phosphorylation site (14E) and to the kinase domain (15E) of the receptor. Western blot and immunoprecipitation analysis of the $_i$EGFR revealed a 155-kd protein which is significantly smaller that the 170-kd human EGF receptor ($_h$EGFR) derived from A431 cells (Cohen *et al.*, 1982). The $_h$EGFR is an extensively glycosylated protein (Cummings *et al.*, 1985; Mayes and Waterfield, 1984) and we postulated that the difference in molecular size between $_i$EGFR and $_h$EGFR is due to differential glycosylation. Thus tunicamycin which inhibits N-linked glycosylation (Tkacz and Lampen, 1975) and monensin which blocks transport of glycoproteins in the Golgi and hence inhibits terminal oligosaccharide processing (Alonso-Caplen and Compans, 1983) were used to determine the effect of glycosylation on the size of the EGF receptor in SF9 cells. Insect and A431 cells were metabolically labelled with [^{35}S]methionine with or without monensin or tunicamycin. The results of SDS–PAGE analysis are shown in Figure 4 and demonstrate that in cells pre-treated with tunicamycin, the polypeptide immunoprepcipitated by Mab R_1 from both insect and A431 cells was 138 kd. Cells pre-treated with monensin contained a 160-kd immunoprecipitable protein in A431 cells and a 155-kd protein in insect cells. Monesin therefore does not influence the size of the human receptor in insect cells, suggesting that, in these cells, the oligosaccharide processing which takes place in A431 cells, does not occur. This is further supported by studies of Endoglycosidase H digestion of the $_h$EGFR and $_i$EGFR. This enzyme hydrolyses core di-*N*-acetylchitobiose linkage in various high mannose glycopeptides. Results shown in Figure 5 illustrate that the human EGF receptor expressed in insect cells was essentially completely hydrolysed by this enzyme, reducing the apparent molecular size by SDS–PAGE from 155 kd to 138 kd (Figure 5 tracks b). Similar treatment of the receptor derived from A431 cells however resulted only in partial side chain hydrolysis reducing the size to 160 kd (Figure 5 tracks a). This supports results from the metabolic studies obtained using monensin and tunicamycin and shows that, in contrast to the $_h$EGFR which contains complex carbohydrate, $_i$EGFR has only high mannose type oligosaccharides which are N-linked to the 138-kd core polypeptide.

Receptor function

Binding affinity studies with the receptor of intact A431 cells revealed two binding affinities of K_d 3.7×10^{-10} M and 3.1×10^{-9} M similar to the results previously reported by Gullick *et al.* (1984). In A431 cell detergent lysates the binding affinity fell to 2.9×10^{-7} M. Analysis of insect

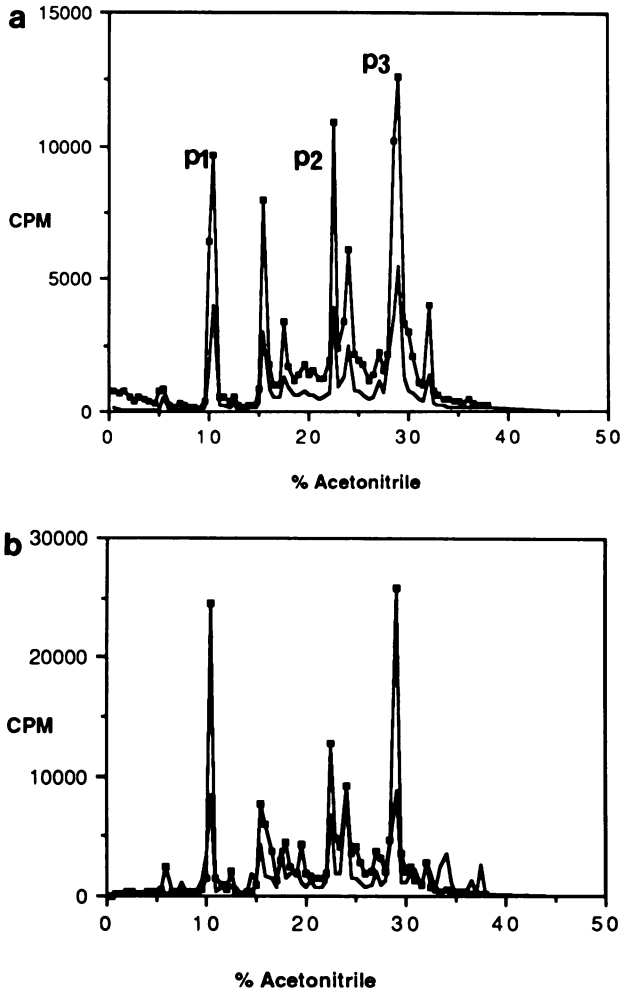

Fig. 7. Tryptic peptide maps of the EGF receptor A431 (**a**) and insect (**b**) cell lysates were incubated with (+) or without (−) EGF then immunoprecipitated with Mab R_1. The immunoprecipitated receptors were then phosphorylated with [γ-^{32}P]ATP 5000 Ci/μmol and analysed by 7% SDS−PAGE. The EGF receptor phospholabelled proteins were digested in the gel with trypsin. the digests were then loaded onto a reverse phase HPLC column and the phosphopeptides eluted in acetonitrile. The phosphopeptide peaks described by Downward *et al.* (1984) are labelled P_1, P_2 and P_3.

cells revealed only a single binding affinity K_d 2 × 10^{-9} M which fell to 2 × 10^{-7} M upon solubilization (Figure 6). Scatchard analysis of EGF binding to intact cells showed that insect cells have 1.2 × 10^5 receptors per cell compared with 2 × 10^6 receptors per cell on A431 cells. Following solubilization in detergent the total receptor levels detected in insect cells were half those found for detergent-treated A431 cells.

The EGF receptor cytoplasmic domain has a tyrosine protein kinase activity which phosphorylates the three major autophosphorylation sites (residues 1173, 1148 and 1068) located close to the receptor carboxyl terminus (Downward *et al.*, 1984). To assess whether the same tyrosine residues were phosphorylated in $_i$EGFR and $_h$EGFR, autophosphorylation was performed in the presence and absence of EGF. The phosphorylated protein was analysed by SDS−PAGE and the labelled receptor digested in the gel with trypsin. The peptides generated were subsequently analysed by HPLC (see Materials and methods). Results shown in Figure 7 show that the $_i$EGFR and $_h$EGFR generated similar phosphopeptides. However, whereas

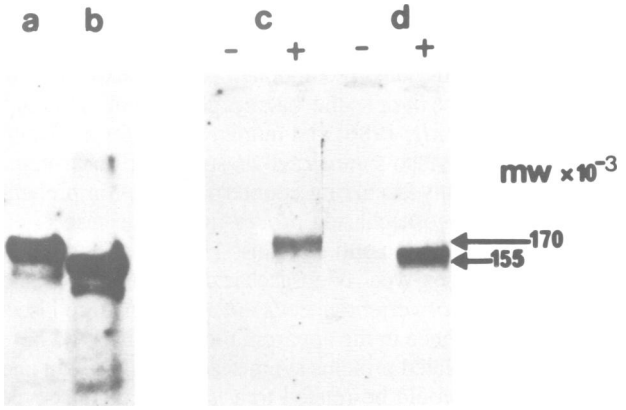

Fig. 8. Assessment of the kinase activity. Equal aliquots of A431 and SF9 cell lysates used for autophosphorylation were immunoblotted using anti-15E antiserum. Equal volumes of cell lysates were incubated with (+) or without (−) EGF. Immunoprecipitates of these lysates were then autophosphorylated and analysed by SDS−PAGE. Autoradiographs of the immunoblots, (**a**) A431 cell lysate (**b**) SF9 cell lysate; and the polyacrylamide gels, (**c**) A431 cell lysate (**d**) SF9 cell lysate; were then analysed by scanning densitometry to obtain a semiquantitative assessment of the kinase activity of the native and recombinant receptors.

Downward *et al.* (1984) demonstrated only three distinct phosphopeptides, the data presented here reveal at least four.

In order to quantitate the specific activity of the receptor kinase it is important to measure the amount of EGF receptor present. This was achieved here by immunoblotting aliquots of cell lysate. (Figure 8; see Materials and methods). Autoradiographs of the blots were scanned to determine the intensity of the 170-kd ($_h$EGFR) and 155-kd ($_i$EGFR) receptor protein bands. Autoradiographs of the autophosphorylated EGF receptor were also quantitated by scanning densitometry. These data allowed a direct relative assessment of the V_{max} to be made for the receptors isolated from the two different cell lines (Figure 8). This analysis of scanned immunoblots and autoradiographs reveals that for similar EGFR content of cell lysates (Figure 8 tracks a and b) the extent of ligand stimulated autophosphorylation is similar for both the $_h$EGFR and $_i$EGFR (Figure 8 tracks c and d).

Discussion

To produce large quantities of normal and mutant recombinant EGF receptor protein for projected biochemical and structural studies, the cDNA encoding the normal human EGF receptor was expressed in insect cells using the baculovirus system. We report here the initial characterization of this protein.

Immunofluorescence analysis of intact cells, using antibodies directed against the external and cytoplasmic domains of the receptor, suggested that insect cells infected with a recombinant virus encoding the human EGF receptor were able to synthesize and process the EGF receptor polypeptide such that it was inserted in the correct transmembrane orientation in these cells. The recognition of the $_i$EGFR by the monoclonal antibody $EGFR_1$ suggests that the protein, at least in part, has the native conformation as this antibody fails to bind to denatured EGF receptor molecules (Waterfield *et al.*, 1982). The correct membrane orientation of the receptor domains is further confirmed by the demonstration of EGF binding to intact cells.

The molecular size of the expressed protein was 155 kd compared with 170 kd for the human EGF receptor in A431 cells. Other glycosylated recombinant proteins expressed in insect cells, for example the haemagglutinin of influenza virus (Kuroda *et al.*, 1986) and human β-interferon (Smith *et al.*, 1983), are also synthesized as smaller glycoproteins than their naturally occurring counterparts. A comprehensive study of oligosaccharide processing by the insect cell *Ades albopictus* (Hsieh and Robbins, 1984) has shown that the larger 'complex-type' oligosaccharides commonly found in glycoproteins of vertebrate cells are absent in these insect cells. The difference in the apparent mol. wt observed here between glycosylated proteins synthesized in SF9 insect and vertebrate cells could be related to a lack of complex-type oligosaccharide modification. The results shown in Figures 4 and 5 support this hypothesis. In A431 cells, monensin which inhibits the formation of the complex-type oligosaccharides (Hubbard and Ivatt, 1981; Mayes and Waterfield, 1984) had a dramatic effect on the size of the $_{h}$EGFR but very little effect on the size of the $_{i}$EGFR. This suggests that $_{i}$EGFR lacks complex-type oligosaccharides; a conclusion that was subsequently supported by the results obtained from the treatment with Endoglycosidase H. As expected from the study using monensin, Endoglycosidase H completely hydrolysed the oligosacchardes from the $_{i}$EGFR reducing the apparent mol. wt from 155 kd to the size of the core protein 138 kd (Figure 5). Similar treatment of the receptor expressed on A431 cells showed only partial hydrolysis of the glycoprotein. This is consistent with the $_{h}$EGFR having >50% complex-type oligosaccharide chain per receptor molecule (Cummings *et al.*, 1985).

Unfortunately, studies of DNA synthesis are impossible in this baculovirus system as all cells that express the receptor are committed to viral-induced cell lysis. Experiments to measure binding of EGF to intact A431 cells revealed receptors with two distinct affinity states (King and Cuatrecasas, 1982). The high affinity class usually represents 5–10% of the total receptor population in A431 cells (Carpenter, 1987). The optimal receptor occupancy required for the initiation of DNA synthesis has been found to be ~ 5–10% and therefore it has been suggested that the high affinity class of receptor may play a major role in the mitogenic signalling process (Shechter *et al.*, 1978; Carpenter and Cohen, 1979). Insect cells expressing the EGF receptor exhibit only a single low affinity class of receptor. Following detergent solubilization of A431 cells, 'high affinity' A431 receptors were lost and both A431 and insect solubilized EGF receptors showed a common single class of binding sites with an affinity for EGF of 2×10^{-7} M. This difference with intact cells in the binding affinities for EGF between the native and recombinant receptor proteins may result from their different glycosylation pattern; conceivably this may induce changes in receptor conformation or aggregation required to form 'high affinity' binding sites (Yarden and Schlessinger, 1987a,b). However, other physico-chemical changes such as an alteration in membrane fluidity or phosphorylation of the amino acid residue threonine 654 in the insect derived receptor may be responsible (Downward *et al.*, 1985; Decker, 1984; Miller *et al.*, 1983). The role of two affinity classes of EGF receptor has not been established (Carpenter, 1987).

It should be noted that purification of the EGF receptor entails detergent solubilization of the plasma membrane. This procedure reduces the receptor's affinity for EGF to a single class at least one order of magnitude lower than the lowest affinity class exhibited in whole cells. However, solubilized receptor with low affinity EGF binding still demonstrates ligand-induced autophosphorylation. The presence of only a single affinity class of EGF receptor produced by the insect cells is not therefore disadvantageous as the $_{i}$EGFR obtained from detergent-solublized SF9 cells behaves in every way similar to $_{h}$EGFR derived from detergent-treated A431 cells. This system can therefore be utilized on a large scale for the purpose of obtaining large quantities of receptor for reconstitution (Panayotou *et al.*, 1985), biochemical and biophysical studies.

The insect receptor tryptic phosphopeptides are very similar to those obtained from the EGF receptor derived from A431 cells. However, whereas Downward *et al.* (1984) originally described three phosphorylated peptides we have reproducibly identified at least four using similar methodology (J.Hsuan, personal communication). The fourth peak is observed in both the A431 cell- and insect cell-derived receptors and the location of this putative fourth phosphorylated tyrosine residue is at present under investigation.

The human EGF receptor expressed in infected SF9 insect cells shows a ligand-stimulated autophosphorylation similar to that of the receptor protein derived from A431 cells. Thus although the recombinant receptor has a different mobility on SDS–PAGE, it binds EGF and has a ligand-stimulated protein kinase activity. Compared to the expression of polyhedrin (1200 mg/l) the level of EGF receptor expression in the baculovirus system is low (1 mg/l). It is, however, comparable to the expression of other recombinant proteins in this system, e.g. 1 mg/l for c-*myc* and 10–20 mg/l for interleukin 2 (Summers and Smith, 1985). The reduced expression of recombinant protein compared to polyhedrin protein could be explained by two mechanisms. Firstly, it is known that mRNA sequences near the AUG initiation codon for protein synthesis have a profound effect on the level of mRNA translation (Kozak, 1986). Retention of the coding sequences of the initial amino acids of the polyhedrin gene seems to result in greater expression of the recombinant protein than those constructs which lack those sequences flanking the polyhedrin AUG (Matsuura *et al.*, 1987; Rohrman, 1986). In our present vector the cDNA of the EGF receptor, which includes 20 bp from the polylinker of pUC18, is inserted −8 bp 5′ to the polyhedrin ATG codon. We are now constructing a new vector in an attempt to increase our yield of $_{i}$EGFR. This vector will contain none of the polylinker or non-coding sequences present in pAc373-EGFR but will retain the nucleotides encoding for the first two amino acids of the polyhedrin peptide in direct continuity with the coding sequencing of the cDNA for the EGF receptor. A second reason for the low level expression recombinant proteins compared to polyhedrin protein may be a result of a codon usage of the foreign cDNA which does not reflect the tRNA population available in the infected insect cell (Bennetzen and Hall, 1982).

Despite the lower levels of expression of recombinant proteins compared to polyhedrin, the baculovirus system is still

capable of producing adequate quantities of functional EGF receptor protein for physico-chemical analysis from basic tissue culture techniques.

Materials and methods

Cells

The insect cell line *S. frugiperda* SF9 (ATCC accession number CRL1711) was propagated at 27°C in Grace's Medium (Gibco) modified with lactalbumin hydrolysate 3.3 g/l and yeastolate 3.3 g/l (Difco) and contained 10% fetal calf serum (FCS). During infection with the virus the cells were cultured in the above with the addition of gentamycin sulphate 50 μg/ml and amphotericin 2.5 μg/ml (Gibco).

Viral and plasmid DNA

Wild-type AcNPV and plasmid Ac373 were the kind gift of Max Summers of A & M College, Texas. The transfer vector pAc373 (Figure 1) contains the baculovirus polyhedrin promoter and a unique *Bam*HI site at −8 bp from the polyhedrin ATG sequence (Smith *et al.*, 1985). Plasmid pUC18-EGFR contains the full length cDNA of the human EGF receptor (Ullirch *et al.*, 1984) cloned into the polylinker of pUC18 (Figure 1).

To construct the recombinant baculovirus vAc-EGFR the segment of DNA corresponding to full length EGF receptor (constructed in this laboratory from three overlapping partial clones) was removed from pUC18-EGFR by first digesting with *Xba*I, filling in with Klenow fragment, and then subsequently digesting with *Kpn*I. This segment was then transferred into the baculovirus polyhedrin plasmid pAc373 which had been digested with *Bam*HI filled in with Klenow fragment and later digested with *Kpn*I. The resulting construct pAc373-EGFR therefore contained all but eight nucleotides of the polyhedrin 5′-untranslated leader sequence fused to 20 nucleotides from the polylinker of pUC18 in continuation with 17 nucleotides of the receptor 5′-non-coding sequence. pAc373-EGFR was subsequently co-transfected with wild-type AcNpV total viral DNA into SF9 cells. (Smith *et al.*, 1983; Miyamoto *et al.*, 1985). *In vivo* recombination events yielded a small proportion of recombinant viruses with the EGF receptor cDNA incorporated into the original viral genome. A recombinant baculovirus was identified in infected insect cell monlayers by DNA hybridization with the cDNA of the EGF receptor. The recombinant virus was the plaque purified by screening for the occlusion negative (polyhedrin negative) phenotype (Summers and Smith, 1987).

Detection of expression of recombinant protein

Purified recombinant virus was used to infect monolayers of cells adherent to poly-L-lysine (Sigma) treated coverslips. At 24, 36, 48, 60 and 72 h post-infection the coverslips were washed in PBSA + 10% FCS and then treated for 15 min with 5% formaldehyde with or without 0.2% Triton X-100. The presence of EGF receptor protein was detected using immunofluorescence with the aid of the monoclonal antibodies R1 and F4 (20 μg/ml) (Gullick *et al.*, 1986) which recognize respectively the external and internal domains of the receptor. Binding was detected by a fluorescein-conjugation goat anti-mouse IgG (Cappel). The coverslips were mounted in a 10% solution of 1.4 diazobicyclo (-222-) octane (Sigma) in glycerol (50%) and examined under UV light. The antibodies used to recognize the various domains of the EGF receptor have been previously reported (Gullick *et al.*, 1985, 1986) and are summarized in Figure 2(i).

EGF binding to whole insect cells

Cells were removed from the dish by washing in Eagle's buffered salt solution, 25 mM Hepes, 0.5% BSA, pH 7.5 (binding buffer). Aliquots (4×10^5 cells) were incubated in a final volume of 500 μl with [^{125}I]EGF (120 pg) and increasing concentrations of unlabelled EGF (0−100 ng) for 4 h at 4°C. The cells were then centrifuged at 4°C and washed twice in binding buffer before being counted in an autogamma spectrometer. The data was corrected for non-specific binding (always < 10% total counts) and analysed by the method of Scatchard (1949).

EGF binding to solubilized insect cells

The cells were solubilized in lysis buffer (25 mM benzamidine, 100 mM Hepes pH 7.4, 5 mM EGTA, 150 mM NaCl, 0.1% (w/v) BSA, 1% (v/v) Nonidet P-40 to which 0.1% phenylmethylsulphonyl fluoride (PMSF) 50 μg/μl, leupeptin 25 mg/ml, aprotonin 10 mg/ml, were added) and cleared at 10 000 *g* for 10 min at 4°C. A final reaction volume of 40 μl contained 40−100 μg of cell lysate, 120 pg [^{125}I]EGF and increasing concentrations of unlabelled EGF (2.5−100 ng) and was incubated for 1 h at 24°C. At this time 10 μl EGF-R_1 antibody (0.2 mg/ml) was added for 10 min at 4°C followed by 10 μl 0.24% γ-globulin and 50 μl polyethyleneglycol (PEG) for a further 10 min at 4°C. The insoluble material was pelleted and washed in 25% PEG. The data were analysed as above.

Immunoblotting

Proteins were electroblotted on nitrocellulose ('Hybond C' Amersham). Non-specific binding was blocked using 5% Marvel. The blot was then incubated for 4 h at 20°C with anti-15E antiserum, then extensively washed in PBSA, Tween 20 (0.05%) and the bound antibody was then detected using iodinated Protein A (Amersham) (5×10^4 c.p.m./ml).

Immunoprecipitation

Infected SF9 cells (10^7) were washed in PBSA, the cell pellet solublized in lysis buffer and receptor protein immunoprecipitated with antibodies Mab R_1, anti-15E or anti-14E (Waterfield *et al.*, 1982; Gullick *et al.*, 1986). Anti-15E and -14E antisera were used with or without competing peptide antigen.

Isotopic labelling

2.5×10^6 SF9 cells were plated onto 6 cm dishes and infected with the recombinant virus. Thirty hours later the medium was replaced with medium deficient in methionine supplemented with 10% dialysed FCS. After 2 h [^{35}S]methionine (> 800 Ci/mmol, Amersham) was added to a final concentration of 500 μCi/ml. Thirty minutes later the cells were washed and re-cultured in complete medium. The cells were lysed 48 h post-infection. For some experiments 5 μg/ml of tunicamycin (Sigma) or 1 μM monensin (Sigma) (final concentrations) were added at the time of the addition of the deficient medium and maintained until the cells were lysed.

As a control study, A431 cells were labelled with [^{35}S]methionine as described by Mayes and Waterfield (1984).

Endoglycosidase H digestion

A431 cells and SF9 cells were metabolically labelled with [^{35}S]methionine for 4 h and then lysed with detergent. The lysates were then immunoprecipitated with Mab R_1. The immunoprecipitate was then boiled for 2 min in 150 μl 50 mM sodium citrate pH 5.5, 0.1 M β-mercaptoethanol and 0.02% (v/v) SDS; the sample was then centrifuged. 7.5 mU of Endoglycosidase H (Boehringer) was then added to a 50 μl aliquot of the supernatant and incubated at 37°C for 24 h, this reaction mixture was then analysed by SDS−PAGE (Trimble and Maley, 1984).

Autophosphorylation

Lysates of A431 or SF9 cells (10^7 cells/500 μl of lysate) were incubated at 4°C with or without EGF (10^{-7} M) for 10 min. The EGF receptor was then immunoprecipitated as above but after washing, the pellet was suspended in 50 μl of phosphorylation buffer (10 μM ATP, 2 mM Mn^{2+}, 12 mM Mg^{2+}, 100 μM V04, 40 mM Hepes pH 7.4, 5% glycerol, 0.2% Triton-X 100, 150 mM NaCl) containing [γ-^{32}P]ATP (5000 c.p.m./pmol). After 10 min at 4°C the reaction was stopped by the addition of sample buffer. The samples were then run on 7% SDS−PAGE gels, which were dried and exposed to Kodak-X-AR5 film at −70°C using intensifying screens.

Peptide mapping

SF9 cells (12×10^7) were infected, harvested 48 h post-infection and lysed in 7 ml of lysis buffer. EGF (10^{-7} M) was added to 3.5 ml for 10 min at 4°C. EGF receptor with or without EGF was immunoprecipitated and phosphorylated as previously described except that only carrier-free [γ-^{32}P]ATP 5000 Ci/μmol (Amersham) was used. To act as a control two 10 cm confluent dishes of A431 cells were treated in a similar fashion (except reactions contained 10 μM ATP). The phosphorylated immunoprecipitate was then analysed by 7% SDS−PAGE and autoradiographed. The labelled proteins were excized and washed in 0.1 M ammonium bicarbonate followed by three washes in 85% acetone, 5% H_2O, 5% triethylamine, 5% acetic acid. The gel slices were then dried *in vacuo* overnight and re-hydrdated with 0.1 M ammonium bicarbonate before treatment with trypsin (Cooper Biomedical 1 mg/ml) for 18 h at 37°C. The peptides were then loaded directly onto a Vydak C18 reverse phase HPLC column (Vydak P. Box 867 Hesperia CA 92345, 4.6 × 75 mm) equilibrated in 0.08% (v/v) trifluoroacetic acid (TFA, Rathburn, Scotland) and eluted with a gradient of 1−40% acetonitrile at 1 ml/min and 0.5 ml fractions were collected. A Hewlett Packard 10/90 M was used with automated injection.

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References

Alonso-Caplen,F.V. and Compans,R.W. (1983) *J. Cell Biol.*, **97**, 659–668.
Bennetzen,J.L. and Hall,B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
Carpenter,G. (1987) *Annu. Rev. Biochem.*, **56**, 881–914.
Carpenter,G. and Cohen,S. (1976) *J. Cell. Biol.*, **71**, 159–171.
Chinkers,M., McKanna,J.A. and Cohen,S. (1979) *J. Cell. Biol.*, **83**, 260–265.
Chinkers,M., McKanna,J.A. and Cohen,S. (1981) *J. Cell. Biol.*, **88**, 422–429.
Cohen,S., Ushiro,H., Stoscheck,C. and Chinkers,M. (1982) *J. Biol. Chem.*, **257**, 1523–1531.
Cummings,R.D., Mangelsdorf-Soderquist,A. and Carpenter,G. (1985) *J. Biol. Chem.*, **260**, 11944–11952.
Decker,S.J. (1984) *Mol. Cell Biol.*, **4**, 1718–1724.
Downward,J., Parker,P. and Waterfield,M.D. (1984) *Nature*, **311**, 483–485.
Downward,J., Waterfield,M.D. and Parker,P.J. (1985) *J. Biol. Chem.*, **260**, 14538–14546.
Fabricant,R.N., DeLarco,J.E. and Todaro,G.J. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 565–569.
Gullick,W.J., Downward,J.H., Marsden,J.J. and Waterfield,M.D. (1984) *Anal. Biochem.*, **141**, 253–261.
Gullick,W.J., Downward,J., Parker,P.J., Whittle,N., Kris,R., Schlessinger,J., Ullrich,A. and Waterfield,M.D. (1985) *Proc. R. Soc. Lond B.*, **226**, 127–134.
Gullick,W.J., Marsden,J.J., Whittle,N., Ward,B., Bobrow,L. and Waterfield,M.D. (1986) *Cancer Res.*, **46**, 285–292.
Hsieh,P. and Robbins,P.W. (1984) *J. Biol. Chem.*, **259**, 2375–2382.
Hubbard,S.C. and Ivatt,R.J. (1981) *Annu. Rev. Biochem.*, **50**, 555–583.
Hunter,T. (1984) *Nature*, **311**, 414–416.
King,A.C. and Cuatrecasas,P. (1982) *J. Biol. Chem.*, **257**, 3053–3060.
Kozak,M. (1986) *Cell*, **44**, 283–292.
Kurodo,K., Hauser,C., Rott,R., Klenk,H.D. and Doerfler,W. (1986) *EMBO J.*, **5**, 1359–1365.
Matsuura,Y., Possee,R.D., Overton,H.A. and Bishop,D.H.L. (1987) *J. Gen. Virol.*, **68**, 1233–1250.
Mayes,E.L. and Waterfield,M.D. (1984) *EMBO J.*, **3**, 531–537.
Miller,L.K., Adang,M.J. and Browne,D. (1983) *J. Virol.*, **46**, 275–278.
Miyamoto,C., Smith,G.E., Farrell-Towt,J., Chizzonite,R., Summers,M.D. and Ju,G. (1985) *Mol. Cell. Biol.*, **5**, 2860–2805.
Moolenaar,W.H., Yarden,Y., de Laar,S.W. and Schlessinger,J. (1982) *J. Biol. Chem.*, **257**, 8502–8506.
Panayotou,G.N., Magee,A.I. and Geisow,M.J. (1985) *FEBS Lett.*, **183**, 321–325.
Rohrman,G.F. (1986) *J. Gen. Virol.*, **67**, 1499–1513.
Scatchard,G. (1949) *Ann. N.Y. Acad. Sci.*, **51**, 660–675.
Shechter,Y., Hernaez,L. and Cuatrecasas,P. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 5788–5791.
Schlessinger,J. (1980) *Trends Biochem. Sci.*, **5**, 210–214.
Smith,G.E., Summers,M.D. and Fraser,M.J. (1983) *Mol. Cell. Biol.*, **3**, 2156–2165.
Smith,G.E., Ju,G., Ericson,B.L., Moschera,J., Lahm,H.-W., Chizzonite,R. and Summers,M.D. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8404–8408.
Staros,J.V., Cohen,S. and Russo,M.W. (1985) In Cohen,P. and Houslay,M.D. (eds) *Molecular Mechanisms of Transmembrane Signalling*. Elsevier, Amsterdam/New York, pp. 253–278.
Summers,M.D., Smith,G.E. (1985) In Fields,B., Martin,M.A. and Kamley,D. (eds), *Genetically-altered Viruses and the Environment*. The Banbury Report, Cold Spring Harbor Laboratory Press, Vol. 22, pp. 319–328.
Summers,M.D. and Smith,G.E. (1987) In Summers,M.D. and Smith,G.E. (eds) *A Manual of Methods for Baculovirus Vectors and Insect Culture Procecures*. Texas Exp. Station Bulletin No. 1555.
Tkacz,J.S. and Lampen,J.O. (1975) *Biochem. Biophys. Res. Commun.*, **65**, 248–257.
Trimble,R.B. and Maley,F. (1984) *Anal. Biochem.*, **141**, 515–522.
Ullrich,A., Coussens,L., Hayflick,J.S., Dull,T.J., Gray,A., Tam,A.W., Lee,J., Yarden,Y., Libermann,T.A., Schlessinger,J., Downward,J., Mayes,E.L., Whittle,N., Waterfield,M.D. and Seebury,P.H. (1984) *Nature*, **309**, 418–425.
Waterfield,M.D., Mayes,E.L.V., Stroobant,P., Bennett,P.L.P., Young,S., Goodfellow,P.N., Banting,G.S. and Ozanne,B. (1982) *J. Cell. Biochem.*, **20**, 149–161.
Yarden,Y. and Schlessinger,J. (1987a) *Biochemistry*, **26**, 1434–.1442.
Yarden,Y. and Schlessinger,J. (1987b) *Biochemistry*, **26**, 1443–1451.

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