Expression of epidermal-growth-factor receptor in the K562 cell line by transfection

Altered receptor biochemistry

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The epidermal-growth-factor (EGF) receptor was expressed in the human erythroleukaemic cell line K562 by transfection of the receptor cDNA. EGF-receptor biochemistry appears altered in the K562 transfectants. Autophosphorylation of the K562 receptor is not stimulated substantially by EGF. Tyrosine kinase activity of the receptor is high in the absence of EGF, whereas receptor affinity for EGF is low. K562 cells are shown to lack mRNA for transforming growth factor α (TGF α). Therefore autocrine stimulation of the K562 receptor, at least by TGF α , does not explain the observed receptor biochemistry. The K562 receptor is phosphorylated at a single major site in intact cells, a threonine residue that may be Thr-669. Possible mechanisms of regulation of the EGF receptor in the K562 transfectants are discussed.

INTRODUCTION

The EGF receptor is a 170 kDa cell-surface glycoprotein that consists of an external EGF binding region, a single transmembrane segment and two cytoplasmic domains, which are a protein-tyrosine kinase and a C-terminal region containing the major autophosphorylation sites (Ullrich *et al.*, 1984; Downward *et al.*, 1984; Gullick *et al.*, 1985). The receptor tyrosine kinase is required for cell proliferation in response to EGF (Chen *et al.*, 1987; Honegger *et al.*, 1987). EGF binding activates the kinase and increases autophosphorylation of the receptor *in vivo*, predominantly at Tyr-1173 near the C-terminus (Downward *et al.*, 1984; Hsuan *et al.*, 1989).

The human erythroleukaemic cell line K562 normally lacks both EGF receptors and histocompatibility antigens HLA-A,B (Drew *et al.*, 1977; Waterfield *et al.*, 1982), but expression of HLA-A,B is inducible with interferon γ (IFN γ ; Sutherland *et al.*, 1985). We expressed the EGF receptor in K562 cells by transfection of the receptor cDNA. There is evidence for interaction of the EGF receptor with major histocompatibility complex class I antigens (HLA-A,B; Schreiber *et al.*, 1984). Our aim was to examine whether interaction with HLA-A,B antigens affects EGF-receptor function. In addition, we wanted to investigate expression of the EGF receptor in an erythroid cell line. We find that EGF-receptor biochemistry is altered in the K562 transfectants irrespective of HLA-A,B expression.

MATERIALS AND METHODS

Transfection and cell culture

The K562 cell line was obtained from the American Type Culture Collection. Cells were transfected with the EGF receptor

cDNA by electric-field-mediated transfection as described previously (Potter *et al.*, 1985; Allen *et al.*, 1986). Transfectants were selected and grown in the presence of the neomycin analogue G418 (0.75 mg/ml). Medium was RPMI 1640 with glutamine, penicillin, streptomycin (pen/strep) and 10% (v/v) fetal-bovine serum (FBS). Transfectants were subcloned by limiting dilution and confirmed as single clones by fluorescence-activated cell-sorting (FACS) analysis with monoclonal antibody (mAb) EGFR1 (results not shown).

EGF binding

Cells were washed once in Earle's buffered salt solution (EBSS)/0.5% BSA/25 mM-Hepes, pH 7.4 (binding buffer). Portions of cells (8×10^6) were incubated in 500 μ l of binding buffer with 125 I-EGF (0.45 ng; 100 μ Ci/ μ g) and a range of concentrations of unlabelled EGF (0–10 μ g) for 4 h at 4 °C. Cells were centrifuged, washed twice in binding buffer at 4 °C and counted for radioactivity in a γ -radiation counter. Data was corrected for non-specific binding (always < 10% total counts) and analysed by the method of Scatchard (1949).

Tunicamycin treatment

Cells (10^7) were incubated in 5 ml of methionine-free, Dulbecco's modified Eagle's medium (DMEM), 2% dialysed FBS and pen/strep for 2 h in the presence or absence of tunicamycin (5 μ g/ml) (Sigma), then [35 S]methionine was added to 100 μ Ci/ml. After 4 h the cells were washed with Ca $^{2+}$ - and Mg $^{2+}$ -free phosphate-buffered saline. Immunoprecipitation with mAb EGFR1 was as described previously (Clark *et al.*, 1988), before analysis by SDS/7.5%-(w/v)-PAGE.

Abbreviations used: EGF, epidermal growth factor; TGF α , transforming growth factor α ; IFN γ , interferon γ ; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting; FBS, fetal-bovine serum; EBSS, Earle's balanced salt solution; DMEM, Dulbecco's modified Eagle medium; pen/strep, penicillin/streptomycin.

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RNA analysis

Polyadenylated [poly(A)⁺]RNA was isolated as described by Seed & Aruffo (1987). RNAs were transferred to GeneScreen filters and filters hybridized as described previously (Allen *et al.*, 1986). The probes for TGF α and β -tubulin mRNAs were isolated from cDNA clones essentially as described by Derynck *et al.* (1987) and Hall *et al.* (1983) respectively.

Autophosphorylation reactions

Membranes were prepared and autophosphorylation reactions performed essentially as described by Pepinsky & Sinclair (1986). Membranes [40 μ g of protein in 20 mM-Hepes (pH 7.4)/2 mM-MgCl₂/10 μ M-Na₃VO₄] were preincubated with or without 100 nM-EGF for 10 min, then incubated with 5 μ M-ATP plus 30 μ Ci of [γ -³²P]ATP for 10 min at 4 °C. Reactions were analysed on SDS/7.5%-PAGE. In addition, autophosphorylation reactions using MnCl₂ instead of MgCl₂, or both MnCl₂ and MgCl₂, were carried out (results not shown). Autophosphorylation of the K562 receptor was not stimulated by EGF under any conditions tested.

Receptors were immunoprecipitated with mAb EGFR 1, and autophosphorylation reactions in immunoprecipitates were carried out as described by Clark *et al.* (1988). Immunoprecipitates were incubated in 50 mM-Hepes (pH 7.4)/150 mM-NaCl/2 mM-MnCl₂/12 mM-MgCl₂/10 μ M-Na₃VO₄/0.2% Triton X-100/10 μ M-ATP/[γ -³²P]ATP (3 μ Ci) for 10 min at 4 °C.

Determination of kinase activity

Plasma membranes were prepared by the method of Thom *et al.* (1977) and the protein content measured. Membranes, 20 μ g of protein in 100 μ l of 25 mM-Hepes (pH 7.4)/150 mM-NaCl/5% (v/v) glycerol/0.2% Triton X-100/100 μ M-Na₃VO₄/2 mM-MnCl₂, were incubated with or without 500 ng of EGF for 10 min at 4 °C, then 100 μ l of 1 mM-RRsrc peptide and 33 μ M-[γ -³²P]ATP (0.34 μ Ci/nmol), was added. The RRsrc peptide contains the autophosphorylation site of p60^{v-src} plus two arginine residues at the N-terminus; the sequence is RRLIEDAEYAARG (Downward *et al.*, 1985). After 0, 1, 2.5, 5 and 10 min at 4 °C, 25 μ l samples were spotted on to P81 filters and placed in 30% (v/v) acetic acid/0.5% (v/v) phosphoric acid. The filters were washed twice in acetic acid plus phosphoric acid, once in 95% ethanol, dried, and the adsorbed radioactivity was counted. The rate of incorporation of ³²P into the RRsrc peptide was calculated. The same membranes were also subjected to SDS/7.5%-PAGE. The gels were Western-blotted and probed with antiserum anti-15E, specific for the receptor tyrosine kinase, as described previously (Clark *et al.*, 1988). Autoradiographs were scanned on an LKB densitometer to estimate in arbitrary units the amount of receptor protein present in each membrane preparation. The linearity of receptor quantification was checked; in addition, the loading of A431 and 11.7 membranes on SDS/PAGE was adjusted so that the levels of receptor were similar.

Analysis of membrane protein phosphorylation by SDS/PAGE, using the same kinase conditions as described above for peptide phosphorylation, showed that the EGF receptor was the only major phosphorylated protein present in both A431 and 11.7 membranes; no significant protein phosphorylation was detectable in K562 membranes (results not shown). In addition, experiments using purified recombinant lipocortin 1 (provided by Dr. B. Pepinsky, Biogen Research Corporation) showed that phosphorylation of lipocortin 1 occurred with A431 and 11.7 membranes, but not with K562 membranes (results not shown).

Phosphopeptide mapping

This was performed essentially as described by Downward

et al. (1984). Cells (5×10^7 11.7; 10^7 A431) were incubated for 16 h with 4 mCi of [³²P]orthophosphate in phosphate-free DMEM, 2% dialysed FBS and pen/strep. Phosphoamino acid analysis was performed by partial hydrolysis and thin-layer electrophoresis essentially as described by Ushiro & Cohen (1980).

Tyrosine phosphatase assay

The EGF receptor was immunoprecipitated from A431 cells and autophosphorylated with [γ -³²P]ATP as described by Clark *et al.* (1988). A431 and K562 cell lysates in Triton X-100 were prepared as for isolation of the EGF receptor (Clark *et al.*, 1988). Protein concentration in the lysates was determined by Bradford assay (Bio-Rad), and equal amounts of lysates were used. Purified alkaline phosphatase was obtained from Boehringer. ³²P-labelled EGF receptor was incubated at 25 °C with cell lysates or alkaline phosphatase in the presence or absence of 1 mM-Na₃VO₄. Reactions were terminated by addition of SDS gel sample buffer and heating at 100 °C for 3 min, then subjected to SDS/7.5% PAGE.

RESULTS

Transfection and expression of EGF receptor

K562 cells were transfected with the human EGF-receptor cDNA in the expression vector pLJR (Fig. 1), which contains the selectable marker aminoglycoside phosphotransferase, by electric-field-mediated transfection (Potter *et al.*, 1985; Allen *et al.*, 1986). Cells resistant to G418 were screened by binding assay with the monoclonal antibody (mAb) EGFR1 for surface expression of the EGF receptor (Waterfield *et al.*, 1982). Two independent K562 transfectants, 11.1 and 11.7, which expressed high levels of EGF receptor, were isolated and subcloned. Both 11.1 and 11.7 cells lacked HLA-A,B expression, as determined by binding assay with mAb W6/32, specific for HLA-A,B (results not shown; Barnstable *et al.*, 1978).

The structure of the EGF receptor expressed by the K562 transfectants was examined by labelling cells with [³⁵S]methionine and immunoprecipitating receptor from lysates with mAb EGFR1. The apparent molecular mass of the K562 receptor on SDS/PAGE was lower than the normal 170 kDa of the A431 receptor. To investigate the basis of the size difference, receptors were immunoprecipitated from tunicamycin-treated, [³⁵S]methionine-labelled 11.7 and A431 cells (Fig. 2). The K562 receptor, in the absence of asparagine-linked glycans, has an apparent molecular mass of 138 kDa, the same as the A431 receptor (Mayes & Waterfield, 1984). Therefore the lower mass of the K562 receptor is due to glycosylation different from that in A431 cells.

The number and affinity of EGF receptors expressed by the

Fig. 1. Organization of the EGF receptor cDNA and the AGPT gene in the pLJR vector

The EGF-receptor cDNA was inserted into the endonuclease-BamHI site of the retrovirus vector pLJR (Haley *et al.*, 1989). Vector pLJR is derived from pLJ (also called DOL⁻; Korman *et al.*, 1987), the polyoma early region was replaced with pUC18 sequences containing the ampicillin-resistance gene (J. Morganstern, unpublished work). Abbreviations: MMLV LTR, Moloney-murine-leukaemia-virus long terminal repeat; SV40 E/P, simian-virus-40 early region enhancer/promoter; AGPT, aminoglycoside phosphotransferase gene, which confers resistance to the neomycin analogue G418.

Fig. 2. Immunoprecipitation of EGF receptor from tunicamycin (Tm)-treated cells

The EGF receptor, lacking asparagine-linked glycans, is the band at 138 kDa marked with an arrow. The truncated form of the EGF receptor present in A431 cells gives rise to the bands at 115 and 95 kDa in the absence, and at 68 kDa in the presence, of tunicamycin (Mayes & Waterfield, 1984).

Fig. 3. Scatchard plots of ^{125}I -EGF binding to K562 transfectants

Cells were incubated at 37 °C in the presence (○) or absence (■) of IFN_γ (1000 units/ml; Biogen Research Corp.) for 48 h before ^{125}I -EGF binding assays.

transfectants were determined by binding studies with ^{125}I -EGF. Scatchard analysis of ^{125}I -EGF binding showed that transfectants 11.1 and 11.7 express about 80000 and 250000 EGF receptors per cell respectively, which are all of low affinity [$K_d = (1.5\text{--}3) \times 10^{-8}\text{M}$; Fig. 3]. To determine whether HLA-A,B expression affected receptor affinity, ^{125}I -EGF binding was assayed on 11.1 and 11.7 cells pretreated with IFN_γ . IFN_γ treatment induced expression of approx. 500000 HLA-A,B molecules/per cell (results not shown). Induction of HLA-A,B expression did not alter receptor affinity for EGF. The number of receptors expressed by transfectants 11.1 and 11.7 was reduced slightly by IFN_γ treatment (Fig. 3).

Kinase activity of EGF receptor

Autophosphorylation of the K562 receptor in the presence or absence of EGF was analysed *in vitro* in membranes and in immunoprecipitates (Figs. 4a and 4b). The receptor in trans-

Fig. 4. Autophosphorylation of EGF receptor in membranes, (a), and in immunoprecipitates (b)

In (c) Western-blot analysis with anti-phosphotyrosine serum was carried out as described by Kamps & Sefton (1988).

fectants 11.1 and 11.7 is autophosphorylated, and phosphorylation is not increased significantly by addition of EGF. By contrast, the A431 receptor shows stimulation of autophosphorylation by EGF, as expected (Waterfield *et al.*, 1982). The EGF receptor expressed in COS-1 cells, by transfection of the same receptor cDNA used in the present study, shows normal EGF stimulation of autophosphorylation (Fig. 4c; J. Haley, unpublished work).

The specific activity of the K562 receptor kinase in plasma membranes was measured in the presence or absence of EGF. The rate of phosphorylation of a synthetic peptide containing the autophosphorylation site of p60^{src} (RRsrc peptide) by membranes isolated from the K562 transfectant 11.7 and A431 cells was determined. The amount of receptor protein present in the membranes was quantified by Western-blot analysis and densitometry of autoradiographs. The kinase activity of the receptor in 11.7 cells was stimulated 2-fold by EGF (11.7 activity without EGF was 587, and with EGF, 1021 pmol of ^{32}P incorporated into RRsrc peptide/min per unit of EGF receptor), whereas activity of the A431 receptor was increased 5-fold (A431 activity without EGF was 98, and with EGF, 535 pmol of ^{32}P incorporated into peptide/min per unit of receptor). Strikingly, the basal activity of the 11.7 receptor was as high as that of the A431 receptor when stimulated by EGF.

Consequently we investigated the possibility that the K562 transfectants might synthesize a ligand for the receptor such as TGF_α and thereby constitutively stimulate receptor kinase activity (Sporn & Roberts, 1985). The presence of TGF_α mRNA in the transfectant 11.7 and parental K562 cell lines was tested by Northern-blot hybridization with a human TGF_α cDNA probe (Fig. 5). Control RNA hybridization with a human β -tubulin cDNA probe is also shown. Both 11.7 and K562 cells lack

Fig. 5. Blot hybridization analysis of TGF α and β -tubulin mRNAs

detectable levels of TGF α mRNA, whereas A431 cells contain the 4.5 kb TGF α transcript as previously reported (Derynck *et al.*, 1987). In addition, 11.7 and K562 cells were shown to lack detectable levels of EGF mRNA (results not shown). Therefore the high basal kinase activity of the 11.7 receptor does not result from autocrine stimulation by TGF α or EGF.

Fig. 6. Tryptic-peptide maps of phosphorylated EGF receptor

The peptide containing phosphothreonine is labelled as T. Results are shown as ^{32}P radioactivity (c.p.m.); ACN is acetonitrile. Receptors from cells treated with EGF are shown as continuous lines and without EGF as dotted lines. The tryptic-peptide mapping was performed twice with identical results. P¹ is the peptide containing phosphotyrosine at residue Tyr-1173.

Phosphorylation sites of EGF receptor

To investigate further how activation of the K562 receptor kinase might occur, we analysed the phosphorylation sites of the receptor in intact cells. Receptors were immunoprecipitated from lysates of 11.7 and A431 cells that had been metabolically labelled with [^{32}P]orthophosphate and treated with or without EGF. ^{32}P -labelled receptors were purified by SDS/PAGE, then digested with trypsin. The tryptic phosphopeptides were separated by h.p.l.c. (Fig. 6). The 11.7 receptor is phosphorylated at one major site, a threonine-containing peptide, in both the absence and presence of EGF. The A431 receptor is extensively phosphorylated at the same site in response to EGF. Heisermann & Gill (1988) have identified threonine-669 as the major phosphothreonine residue of the A431 receptor. Comparison of h.p.l.c. profiles suggests that the site phosphorylated in the K562 receptor also may be threonine-669.

The A431 receptor is phosphorylated at the P¹ site of Tyr-1173 in response to EGF. Tyr-1173 was identified previously as the major site of tyrosine autophosphorylation *in vivo* by Downward *et al.* (1984). The absence of detectable P¹ phosphorylation of the 11.7 receptor was initially surprising. However, the 11.7 receptor shows constitutive autophosphorylation *in vitro* at the P¹, P² and P³ sites mapped by Downward *et al.* (1984) (J. J. Hsuan, unpublished work). Therefore we suspected that the lack of P¹ phosphorylation *in vivo* might be due to tyrosine phosphatase activity in K562 cells. This was substantiated by two approaches. First, protein tyrosine phosphatase activity in K562 and A431 cell lysates was assayed using EGF receptor, autophosphorylated *in vitro* in immunoprecipitates with [γ - ^{32}P]ATP, as substrate (Fig. 7). The ^{32}P -labelled receptor was stable in lysis buffer under the assay conditions of 20 min at 25 °C (Fig. 7, lanes 1–3). Purified alkaline phosphatase was used as a positive control to demonstrate complete removal of [^{32}P]phosphate from the receptor (lane 4). This enzyme was partially inhibited by the addition of 1 mM- Na_3VO_4 (lane 5). Both A431 and K562 lysates dephosphorylated the receptor and were completely inhibited by vanadate (lanes 6–8 and 9–11 respectively). However, the K562 lysate contained more than 4-fold higher protein tyrosine phosphatase activity.

Secondly, the presence of phosphotyrosine on the K562 receptor in intact cells was investigated by Western-blot analysis with anti-phosphotyrosine serum (Kamps & Sefton, 1988; Fig. 8). This method is more sensitive than analysis of tryptic phosphopeptides (Fig. 6). Cells were incubated overnight in the presence or absence of Na_3VO_4 , then treated, or not, with EGF. Receptors were immunoprecipitated from cell lysates, separated

Fig. 7. Assay of protein tyrosine phosphatase activity in K562 and A431 cell lysates

Abbreviation: Cip, alkaline phosphatase from calf intestine (Boehringer).

Fig. 8. Western-blot analysis of EGF receptor with anti-phosphotyrosine serum

Cells (11.7 and K562) were incubated with 500 μ M-Na₃VO₄ for 16 h at 37 °C; A431 cells were incubated with 100 μ M-Na₃VO₄. Subsequently cells were treated with EGF (50 ng/ml) for 10 min at 37 °C. Cell viability was 90 % or higher after treatment with Na₃VO₄.

by SDS/PAGE and Western-blotted. Tyrosine phosphorylation of the 11.7 receptor was detected in cells treated with vanadate; addition of EGF does not stimulate this phosphorylation substantially (Fig. 8, lanes 2–5). Basal phosphorylation of the A431 receptor is increased by vanadate treatment, but a further EGF-dependent increase does occur (Fig. 8, lanes 6–9). In summary, these results are consistent with the idea that autophosphorylation of the K562 receptor is reversed by tyrosine phosphatase action in intact cells.

DISCUSSION

We expressed the EGF receptor in the human erythroleukaemic cell line K562 by transfection of the receptor cDNA. EGF-receptor biochemistry appears altered in the K562 transfectants. Basal autophosphorylation of the K562 receptor is readily detected, but is not stimulated substantially by EGF. This was demonstrated in immunoprecipitates, membranes and vanadate-treated cells. The specific activity of the K562 receptor kinase was determined for a peptide substrate. In the absence of EGF, basal kinase activity is high; addition of EGF increases activity only 2-fold. The basis of the difference in EGF stimulation seen for peptide phosphorylation and receptor autophosphorylation in membranes is not clear. It remains possible that a small increase in autophosphorylation is induced by EGF, but is not detected because of the speed of the autophosphorylation reaction. The EGF receptors expressed by the K562 transfectants are all of low affinity for EGF ($K_d \approx 10^{-8}$ M). One explanation for both the low-affinity and high-basal kinase activity might be autocrine stimulation of the K562 receptor. If K562 cells synthesized a ligand for the EGF receptor, potentially this could result in occupation of high-affinity receptors and kinase activation. We have shown that K562 cells lack mRNA for TGF α ; thus autocrine stimulation by TGF α does not explain the observed receptor biochemistry. However, synthesis of another ligand by K562 cells is still possible [e.g. amphiregulin (Shoyab *et al.*, 1989)].

To examine further how regulation of the K562 receptor might occur, we analysed the phosphorylation sites of the receptor in intact cells. The K562 receptor is phosphorylated at a single major site, a threonine residue with a mobility, by reverse-phase h.p.l.c., similar to that of Thr-669, in both the absence or presence of EGF. Countaway *et al.* (1989) have identified a threonine kinase that may control the phosphorylation state of the EGF receptor at threonine-669. They have shown that the kinase is activated by EGF, platelet-derived growth factor and

phorbol ester. This kinase may be activated possibly by another mechanism in K562 cells and cause phosphorylation of the receptor. Whether phosphorylation of threonine-669 either increases kinase activity or decreases affinity for EGF of the K562 receptor is not yet clear. This issue should be resolvable by mutagenesis of threonine-669 and analysis of function of the mutant receptor expressed in K562 cells.

Introduction of the EGF receptor into primary haematopoietic cells or interleukin 3-dependent cell lines resulted in normal receptor function (Khazaie *et al.*, 1988; Pierce *et al.*, 1988; von Ruden & Wagner, 1988). K562, by contrast, is a transformed haematopoietic cell line, a feature of which is expression of the activated tyrosine kinase p210^{bcrl/abl} (Konopka *et al.*, 1984; Ben-Neriah *et al.*, 1986). The oncogenic potential of p210^{bcrl/abl} has been demonstrated by its ability to transform an interleukin 3-dependent cell line (Daley & Baltimore, 1988). Therefore, expression of p210^{bcrl/abl} may be relevant to the altered biochemistry of the EGF receptor in the K562 transfectants. This possibility is of interest for future investigation.

Note added in proof (received 21 September 1990)

After submission of this paper, the amphiregulin cDNA sequence was published (Plowman *et al.*, 1990). By using a synthetic oligonucleotide based on the cDNA sequence as a probe for Northern-blot hybridization, we have shown that the transfectant 11.7 and parental K562 cell lines lack detectable levels of amphiregulin mRNA. Therefore K562 cells do not appear to express any of the three known ligands for the EGF receptor. This suggests that, if autocrine stimulation causes the altered K562 receptor biochemistry, the ligand might be a novel one.

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