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EPIDERMAL GROWTH FACTOR INDUCED TYROSINE PHOSPHORYLATION OF NUCLEAR PROTEINS ASSOCIATED WITH TRANSLOCATION OF EPIDERMAL GROWTH FACTOR RECEPTOR INTO THE NUCLEUS

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Abstract—Treatment of human squamous carcinoma cells (HN5 cells) with epidermal growth factor (EGF) caused a time-dependent increase in tyrosine phosphorylation of six nuclear proteins of molecular mass 166, 140, 117, 95, 86 and 79 kDa. The major tyrosine phosphorylated protein was indistinguishable from the plasma membrane form of the epidermal growth factor receptor and was shown by enzyme linked immunosorbent assay (ELISA) to be translocated into the nucleus from extra-nuclear sites upon ligand stimulation. Using immunoelectron microscopy of both isolated nuclei and whole cells, epidermal growth factor receptor (EGF-R) was found to be associated with the chromatin and, to a lesser extent, with the inner surface of the nuclear membrane. Tyrosine phosphorylation of proteins other than EGF-R was particularly notable in the nucleoli. These observations suggest that EGF-R may exert some of its physiological functions by directly inducing tyrosine phosphorylation of specific nuclear proteins. Translocation of EGF-R to the nucleus may provide a vital link between plasma membrane signalling and gene activation

The EGF-R‡ is a membrane bound glycoprotein whose intrinsic protein tyrosine kinase activity is stimulated upon ligand binding [1]. This initiates a complex series of events within the cell which ultimately leads to cell division. Although the early events in the signal transduction pathway have been examined in some detail (reviewed in Refs. 2 and 3), it is not known how the cytoplasmic signals which occur within minutes of exposure to the ligand are coupled to induction of DNA synthesis which requires cells to be exposed to growth factor for several hours [4]. Nor is it clear how the broadly similar spectrum of second messengers which are generated by binding of a wide range of ligands to their appropriate receptors achieves the specificity of gene activation and cellular response brought about by an individual polypeptide growth factor such as EGF [5].

Recent work linking occupation of IFNα receptors [6] or IFNγ receptors [7] with activation of latent transcription factors by tyrosine phosphorylation and their resultant translocation to the nucleus suggests one mechanism whereby ligand specificity of gene activation may be achieved. Additionally, there is evidence for translocation of some growth factors notably, EGF [8–12], insulin [13], IL-1 [14], FGF [15] and NGF [9] and/or their membrane receptors to the nucleus and, in the case of FGF, this translocation has been shown to be cell cycle specific. Although the role of these molecules within the nucleus remains to be defined, it is possible that they may control activation of specific genes.

The present study was undertaken to determine whether there was a link between EGF-R tyrosine kinase activation and the phosphotyrosine content of nuclear proteins. The cells used in the present experiments were HN5 cells obtained from a human squamous carcinoma cell line [16] which express in excess of 2×10^6 EGF-R on their surface. Using these cells, we have demonstrated the occurrence of EGF-R in nuclei and have defined its morphological location by immunoelectron microscopy. We extend these observations further by demonstrating ligand induced translocation of EGF-R to the nucleus and associated tyrosine phosphorylation of other nuclear proteins.

MATERIALS AND METHODS

Immunoreagents. Primary antibodies used in this study were PY20 (ICN Flow, Irvine, U.K.) which is a biotinylated mouse monoclonal antiphosphotyrosine antibody; EGF-R1, a mouse monoclonal anti EGF-R antibody (obtained from Amersham International Amersham, U.K. and purified by FPLC to remove

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‡ Abbreviations: EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; IFNα, interferon alpha; IFNγ, interferon gamma; FGF, fibroblast growth factor; NGF, nerve growth factor; IL-1, interleukin 1; DMEM, Dulbecco's modified Eagle's medium; PBSI, phosphate-buffered saline with inhibitors; HEPES, hydroxyethylpiperazine ethanesulphonic acid; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulphonyl fluoride; HRP, horseradish peroxidase; DEAE, diethylaminoethyl; HN5, human squamous carcinoma cell line LICR-LON HN5; TGFα, transforming growth factor alpha; PDGF, platelet derived growth factor.

bovine serum albumin) which recognises an epitope in the extracellular domain of EGF-R [17]; MoAb 3138, a mouse monoclonal anti EGF-R antibody (obtained from the Sigma Chemical Co., Poole, U.K.) which recognises an epitope in the intracellular domain of EGF-R [18]; Ab529, a DEAE purified IgG fraction from sheep antiserum raised against affinity purified human EGF-R [the antibody was raised against EGF-R affinity purified from detergent solubilized A431 plasma membranes using EGF-Affigel; it reacts with a 170 kDa protein on western blots of A431 cells and neutralizes mitogenesis induced by EGF or TGFα, but not PDGF or FGF, in human foreskin fibroblasts (A. Richter and D. E. Davies, unpublished observations)].

Cells and cell culture. HN5 cells [16] were cultured in DMEM supplemented with 10% foetal calf serum in 140-mm petri dishes. Experiments were performed with cells at 80% confluence and 2 days after addition of fresh medium.

Isolation of cell nuclei. At the indicated times (see text) after addition of human recombinant EGF, cells were rinsed three times with ice cold PBS containing phosphatase and protease inhibitors (PBSI; 9.2 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.15 M NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 5 μg/mL leupeptin and 1 μg/mL aprotonin). Release of nuclei was initiated in 6 mL of lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 5.5) by the addition of sufficient Zaponin (Coulter Electronics, U.K.) (approximately 20 drops) to cause immediate release of the nuclei (observed by phase contrast microscopy). The nuclei were collected and pelleted by centrifugation through 20% sucrose in PBSI. They were then resuspended in PBSI and counted using a haemocytometer.

Detection of tyrosine phosphorylated proteins by SDS–PAGE and western blotting. Nuclei or whole cells were solubilized in sample buffer [2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% glycerol and bromophenol blue in 0.25 M Tris–HCl pH 6.8] and heated at 95° for 5 min with continuous mixing. Proteins were separated by SDS–PAGE [19] in 7.5% acrylamide gels and then transferred to nitrocellulose membranes (Hybond C, Amersham) by electrophoresis. Immunoblotting was performed after incubating the membrane in PBSI containing 5% non-fat milk powder and 0.5% Tween 20 for 1 hr at room temperature. Proteins phosphorylated on tyrosine were detected using biotinylated anti-phosphotyrosine PY20 antibody at a dilution of 1 in 1600 followed by biotinylated HRP-streptavidin conjugate (1 in 1000, for 1 hr at room temperature in antibody buffer) and ECL detection reagents (both from Amersham International).

Measurement of EGF-R and its degree of tyrosine phosphorylation by ELISA. EGF-R was measured by standard sandwich ELISA protocol using the EGF-R1 monoclonal antibody applied to the plates at 5 μg/mL overnight at 4°, to capture the receptor. All incubation and wash buffers contained 0.1 mM Na_3VO_4, 0.1 mM NaF, and 0.1 mM PMSF. Pelleted nuclei, prepared as described above, or cells (washed in PBSI, scraped and pelleted by centrifugation) were solubilized in 5% Triton X-100, 10% glycerol, 0.1 M Na_3VO_4, 0.1 M NaF, 0.1 M PMSF, 20 mM HEPES, pH 7.4 and diluted such that the detergent concentration was below 2%, and then incubated on the plates for 16 hr at 4°. The EGF-R was detected using 2 μg/mL of Ab529 followed by donkey anti-sheep IgG peroxidase conjugate.

Phosphorylated tyrosine residues on the EGF-R were detected using the biotinylated PY20 antibody at 1 μg/mL and streptavidin-biotinylated HRP conjugate at 1 in 1000 dilution instead of Ab529 and the peroxidase conjugate used above.

Electron microscopy. (1) Immuno-electron microscopy. HN5 cells were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hr at 4°, then scraped from the dish, pelleted and encapsulated in low setting temperature agarose. Nuclei, which were isolated as described above, were fixed, pelleted and encapsulated as described for whole cells. Samples were processed by the progressive lowering of temperature method [20] into Lowicryl HM20 resin using a Leica CS Auto cryo substitution apparatus. The resin was polymerized with UV light for 48 hr at −50°. Sections (90 nm) were picked up on nickel C/Formvar grids and blocked in 10% (v/v) normal goat serum in diluent (0.2 M Tris, 0.5% bovine serum albumin) before incubation with primary antibody. The antibodies used were EGF-R1 at 50 μg/mL, MoAb 3138 at 1 in 200, Ab529 at 50 μg/mL or PY20 (anti-phosphotyrosine) at 1 in 400. The antibodies were diluted in buffers as described for ELISA. Sections were incubated with antibody in a wet box for 15–16 hr at 20°. Secondary antibody, goat F(ab)2 anti-mouse IgG + IgM conjugated to 10 nm gold diluted 1 in 10 or donkey anti-sheep IgG conjugated to 10 nm gold (BioCell) diluted 1 in 40, was applied to sections for 1 hr. Sections were counterstained with uranyl acetate and lead citrate and examined in a Hitachi H7000 TEM.

(2) Scanning electron microscopy (SEM). Nuclei, prepared as described above, were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and washed in 0.1 M cacodylate buffer with 0.23 M sucrose. Samples were then left for 2 hr in 2% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated through graded ethanol and critical point dried in a Balzers CPD030 from ethanol using liquid CO_2 as the exchange medium. Samples were stub mounted and coated with gold paladium using a Polaron E5100 sputtercoater and examined in a Hitachi 5800 field emission scanning electron microscope.

Acid phosphatase assay. Acid phosphatase (EC 3.1.3.2) activity was determined using slight modifications of the method of Ericcson and Trump [21]. These modifications, which were to enable cell or nuclear suspensions to be used, were that the cells were fixed for 10 min in 3% glutaraldehyde in 0.1 M cacodylate and incubations with sodium β-glycerophosphate as substrate were reduced to 5 min at room temperature. Control incubations included the omission of substrate, inclusion of sodium fluoride (10 mM) as inhibitor and omission of the lead nitrate capture agent.

5′-Nucleotidase assay. 5′-Nucleotidase (EC 3.1.3.5) was measured in whole cell extracts or in

isolated nuclei using [^{3}H]AMP as substrate as described by Avruch and Wallach [22].

RESULTS

Investigations using isolated nuclei

To analyse the effect of EGF on nuclear proteins, it was first necessary to establish that the nuclear preparation used was of high quality. A number of protocols for isolation of nuclei were investigated for their ability to yield intact nuclei with minimal contamination by plasma membranes or other cellular organelles. Of these, the Zaponin method was found to be the most effective. Zaponin solubilizes plasma and cytoplasmic membranes and therefore minimizes the risk of contamination of the nuclei with these structures. Light and electron microscopy showed the nuclear preparations to be highly purified and well preserved with the inner membrane intact (Fig. 1). Furthermore, in thin sections of isolated nuclei no cytoskeletal contamination was detected using anti-vimentin antibodies (see below). As a further indicator of contamination, the activity of 5′-nucleotidase was also measured. This was found to be 12.54 ± 1.06 μmol/min/mg protein for whole cell homogenate and 0.012 ± 0.002 μmol/min/mg protein for isolated nuclei, demonstrating that contamination with this plasma membrane marker enzyme was less than 0.1%. In addition, acid phosphatase activity (a lysozomal enzyme) was absent in isolated nuclei; in contrast, in whole cell preparations characteristic acid phosphatase activity could be detected in lysozyme-like structures throughout the cytoplasm. To eliminate the possibility of non-specific association of cytoplasmic proteins with damaged nuclei the integrity of the nuclear membrane was investigated by SEM; nuclei were found to be 98% intact displaying convoluted inner nuclear membrane with regular nuclear pores (Fig. 1c and d).

Having established the purity of nuclei isolated by the Zaponin method, tyrosine phosphorylation of nuclear proteins in response to EGF was examined by western blot analysis of proteins solubilized from isolated nuclei. This revealed a time-dependent increase in the phosphotyrosine content of six proteins of molecular mass 166, 140, 117, 95, 86, and 79 kDa (Fig. 2 a–e). Maximum phosphorylation of the six proteins was observed between 30 and 60 min and then declined towards original levels, or less, by 24 hr. Comparison of the phosphotyrosine profile of the isolated nuclei with that of whole cells treated with EGF for 1 hr (Fig. 2f) showed clear differences in the phosphorylation pattern. However, in both cases the major phosphorylated protein had a molecular mass of around 170 kDa. This was shown to be EGF-R by western blotting (data not shown) and was quantitated by ELISA (see below).

To distinguish between translocation of phosphorylated EGF-R into the nucleus or tyrosine phosphorylation of a resident nuclear EGF-R population, two ELISAs were used to measure EGF-R and EGF-R specific phosphotyrosine in the nuclear preparations. Using the EGF-R phosphotyrosine specific ELISA, a dose-dependent increase in nuclear EGF-R phosphotyrosine content was observed 1 hr after EGF treatment (Fig. 3). At the highest dose of EGF used (100 ng/mL), small changes in nuclear EGF-R phosphorylation were apparent within 2 min of EGF treatment and by 1 hr there was a 3-fold increase in receptor phosphotyrosine content (Fig. 4). This is in marked contrast to whole cells where maximum tyrosine phosphorylation of EGF-R was observed within 2 min of EGF treatment (Fig. 4). Using the EGF-R ELISA, in whole cells EGF-R levels did not change significantly at any time point following treatment with EGF. In isolated nuclei however, EGF-R levels doubled upon EGF treatment indicating that EGF caused translocation of its receptor into the nucleus. As the cells were used in late log phase, the presence of EGF-R in nuclei of untreated cells probably reflects an autocrine effect as, like many other carcinoma cells, HN5 cells synthesize several ligands for EGF-R, namely TGFα, amphiregulin and heparin-binding EGF (N. Solic and D. E. Davies, unpublished observations).

The location of both EGF-R and tyrosine phosphorylated proteins in nuclei was confirmed and further defined by electron microscopy using immunogold labelling. Phosphotyrosine was located both within the chromatin (Fig. 5a) and in the nucleolus where it was particularly apparent (Fig. 5b). EGF-R was localized to the chromatin surrounding the nucleoli (Fig. 5c) and, to a lesser extent, in the vicinity of the inner leaflet of the nuclear membrane (Fig. 5d) close to the nuclear pores. It is noteworthy that both EGF-R and phosphotyrosine labelling were concentrated in particular regions of the nucleus and suggest the presence of specific sites for the interaction of EGF-R and phosphotyrosine-containing proteins in chromatin. The amount of EGF-R and phosphotyrosine associated with representative nuclei was assessed by counting the gold particles of the immunoconjugate. Table 1 shows that the amount of EGF-R was 10 times higher and phosphorylation of tyrosine was increased 4-fold in nuclei isolated from cells following stimulation with 100 ng/mL EGF for 1 hr. As control, anti-vimentin antibodies or anti-phosphotyrosine antibodies pre-adsorbed with phosphotyrosine were applied to sections. In neither case was any gold labelling of nuclei observed.

Investigations with intact cells

To determine if occurrence of EGF-R in isolated nuclei was an artefact due to contamination of nuclei with EGF-R during their preparation, intact HN5 cells were treated with EGF and subjected to immuno-electron microscopy. As previously reported for A431 cells [23], EGF treatment caused a large reduction (around 80%) in plasma membrane EGF-R immunolabelling when compared with untreated cells; this was paralleled by a 4-fold increase in the nuclear content of EGF-R (Fig. 6). The distribution and pattern of labelling was similar using three EGF-R antibodies (EGF-R1, MoAb 3138 and a polyclonal sheep anti-EGF-R) (Fig. 6). Phosphotyrosine labelling was also shown to be redistributed upon EGF treatment. Thus, 1 hr after EGF treatment, there was a 4-fold reduction in

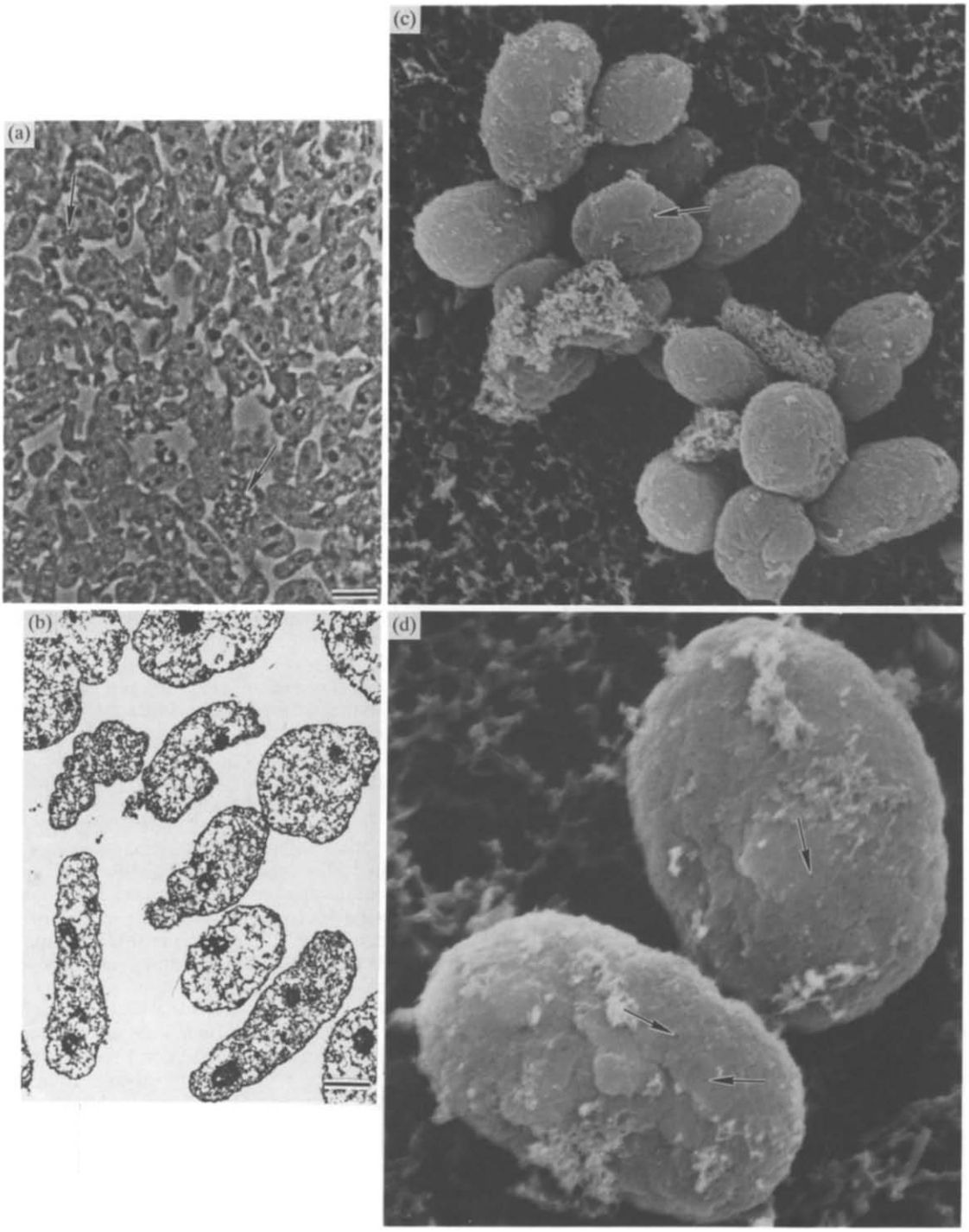

Fig. 1. Light and electron micrographs of purified nuclei isolated from HN5 cells. (a) 1 μm section through nuclei embedded in Epon resin shows the nuclear preparation to be free from contaminating membranes (arrows indicate nuclei in anaphase) and (b) an electron micrograph (80 nm section) showing the inner nuclear membrane to be intact. A surface view visualized by scanning electron microscopy shows undamaged inner membranes which are deeply invaginated (arrows in c), and at higher magnification (d) nuclear pores can be seen (arrows in d). Bar = 6 μm in a, 2.11 μm for b and 167 nm for c and d.

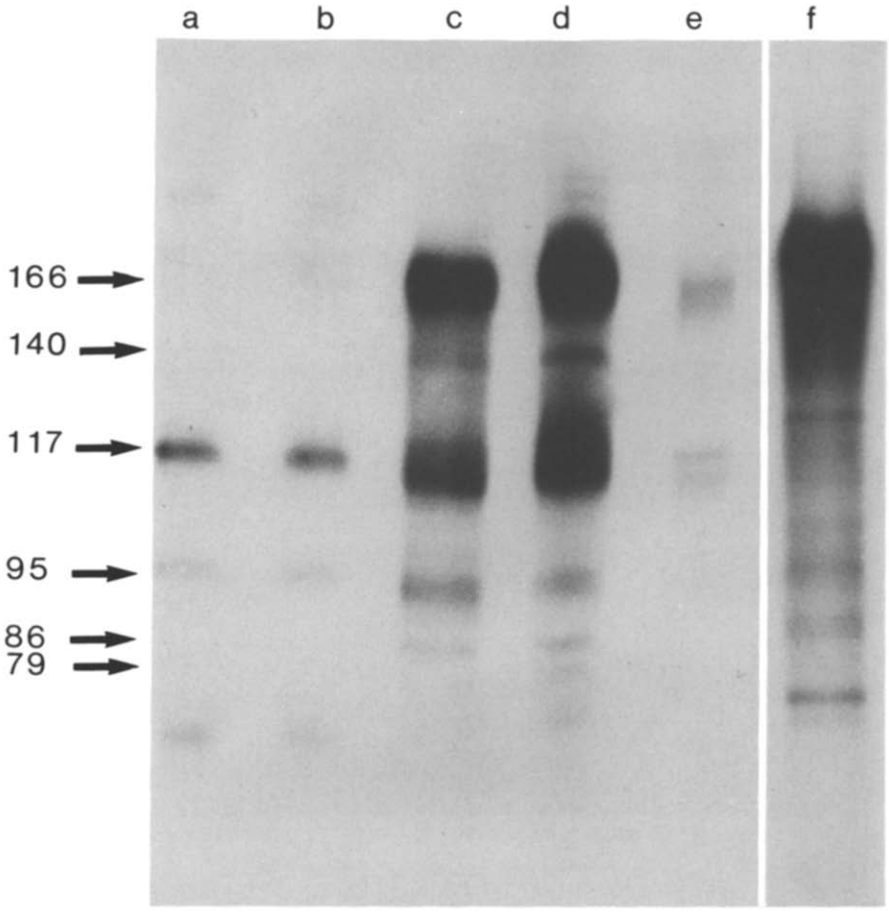

Fig. 2. Appearance of tyrosine phosphorylated proteins in either isolated nuclei before (a) and at 2 min (b), 30 min (c), 1 hr (d), and 24 hr (e) after the cells were treated with EGF (100 ng/mL), or in whole cells 1 hr after EGF (100 ng/mL) treatment (f). The major phosphorylated proteins are indicated by arrows with molecular masses in kDa. Each lane contained an equivalent number of nuclei ($\approx 2 \times 10^4$) (a–e), or 20 μg of whole cell protein (f) solubilized in the same way after scraping cells from the culture dish in PBSI and pelleting.

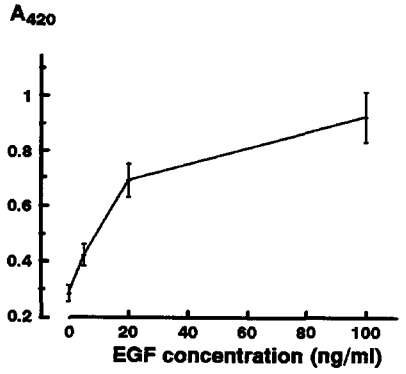

Fig. 3. Dependence of the degree of nuclear EGF-R tyrosine phosphorylation on EGF concentration. HN5 cells were treated with EGF at the concentrations indicated and nuclei isolated and assayed by ELISA as described in the Materials and Methods. Results shown correspond to $\approx 10^7$ nuclei/microtitre well and are means ± SEM of five determinations at each point.

plasma membrane labelling and a doubling in the nuclear phosphotyrosine content. No labelling was seen in mitochondria. As a further control to establish that the translocation of EGF-R to the nucleus was an active process which did not take place in the cold, cells were incubated for 2 hr at 4° prior to fixation. Under these conditions, the majority of both the EGF-R and phosphotyrosine staining was on the plasma membrane, with little nuclear labelling. Following treatment with EGF, no translocation of EGF-R or phosphotyrosine was observed.

Thus, the changes in EGF-R and phosphotyrosine distribution observed in isolated nuclei were paralleled in intact cells under physiological conditions.

DISCUSSION

The activities of key nuclear effector molecules such as transcription factors and tumour suppressor proteins are known to be regulated by serine/threonine phosphorylation (reviewed in Refs. 25

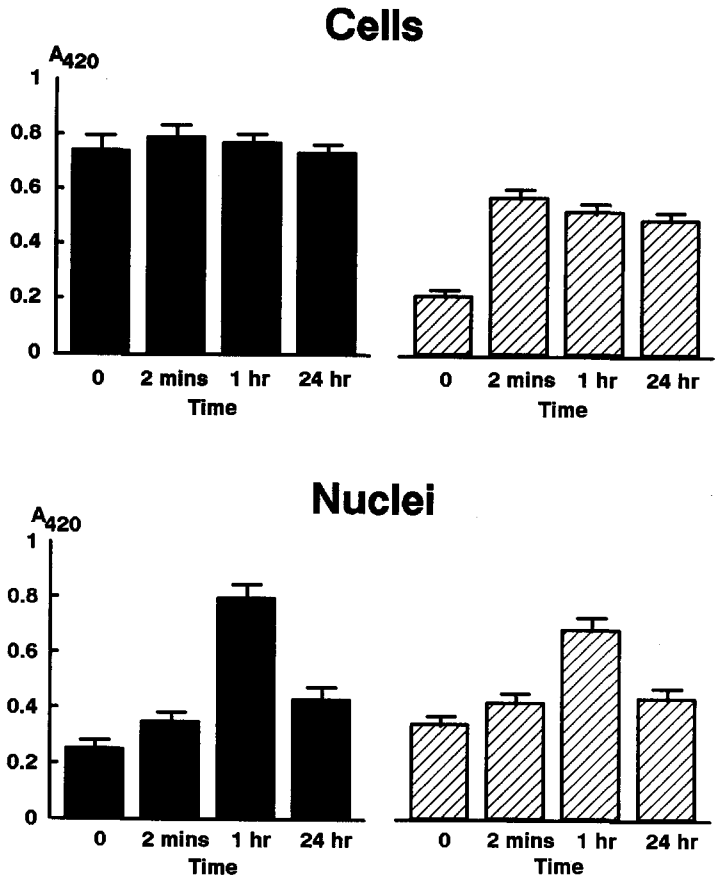

Fig. 4. Time course of the effect of EGF on EGF-R concentration (solid bars) and EGF-R tyrosine phosphorylation (hatched bars) in HN5 cells and isolated nuclei. HN5 cells were treated with EGF at 100 ng/mL for the times indicated and either whole cells or nuclei were prepared and assayed for EGF-R concentration and EGF-R phosphotyrosine content using the ELISAs described in the Materials and Methods. Results shown correspond to 10^5 cells or nuclei from 10^7 cells/microtitre well and are means ± SEM of six determinations for each point.

and 26). In the present study, evidence is presented that EGF induces tyrosine phosphorylation of nuclear proteins and that this may, in part, be catalysed by EGF-R which is translocated to the nucleus upon ligand stimulation. Our observation of EGF-stimulated tyrosine phosphorylation of several nuclear proteins together with the recent report of activation by tyrosine phosphorylation of latent transcription factors regulated by IFNα [6] or IFNγ [7] suggests that another level of control may be exerted by phosphorylation of tyrosine residues. Purification and characterization of the EGF-stimulated tyrosine phosphorylated nuclear proteins will enable their function to be established.

Although the present studies do not distinguish between tyrosine phosphorylation of nuclear proteins catalysed by nuclear tyrosine kinases or their cytoplasmic phosphorylation and translocation into the nucleus as is caused by IFNα [6] or IFNγ [7], either mechanism may be operative. Thus, phosphorylation of resident nuclear proteins may be catalysed by chromatin associated EGF-R. Such a regulatory role might also explain the occurrence of FER [27] and *c-abl* [28] tyrosine kinases in nuclei and the reported DNA-binding capabilities of *c-abl* [29]. Alternatively, as EGF-R is internalized upon ligand stimulation and is routed via the endosomes to the perinuclear region [23], it could catalyse phosphorylation of cytoplasmic factors which then translocate to the nucleus.

There are several claims for nuclear translocation of some membrane receptors and/or their ligands (e.g. IL-1 and its receptor, insulin and its receptor, FGF and NGF) (reviewed in Ref. 30), however, their role in the nucleus remains to be defined. In addition, certain enzymes (e.g. those involved in inositol phospholipid metabolism and protein kinase C) which are considered to play key roles in plasma membrane signalling, also appear to have additional roles at the nuclear level [31]. Localization of EGF-R in nuclei of EGF treated HN5 cells confirms previous reports of the presence of EGF-R in nuclei [10–12] but conflicts with the work of Carpentier *et al.* [23] who failed to detect EGF-R in nuclei by immunoelectron microscopy of whole cells. The failure of Carpentier *et al.* to detect EGF-R in nuclei

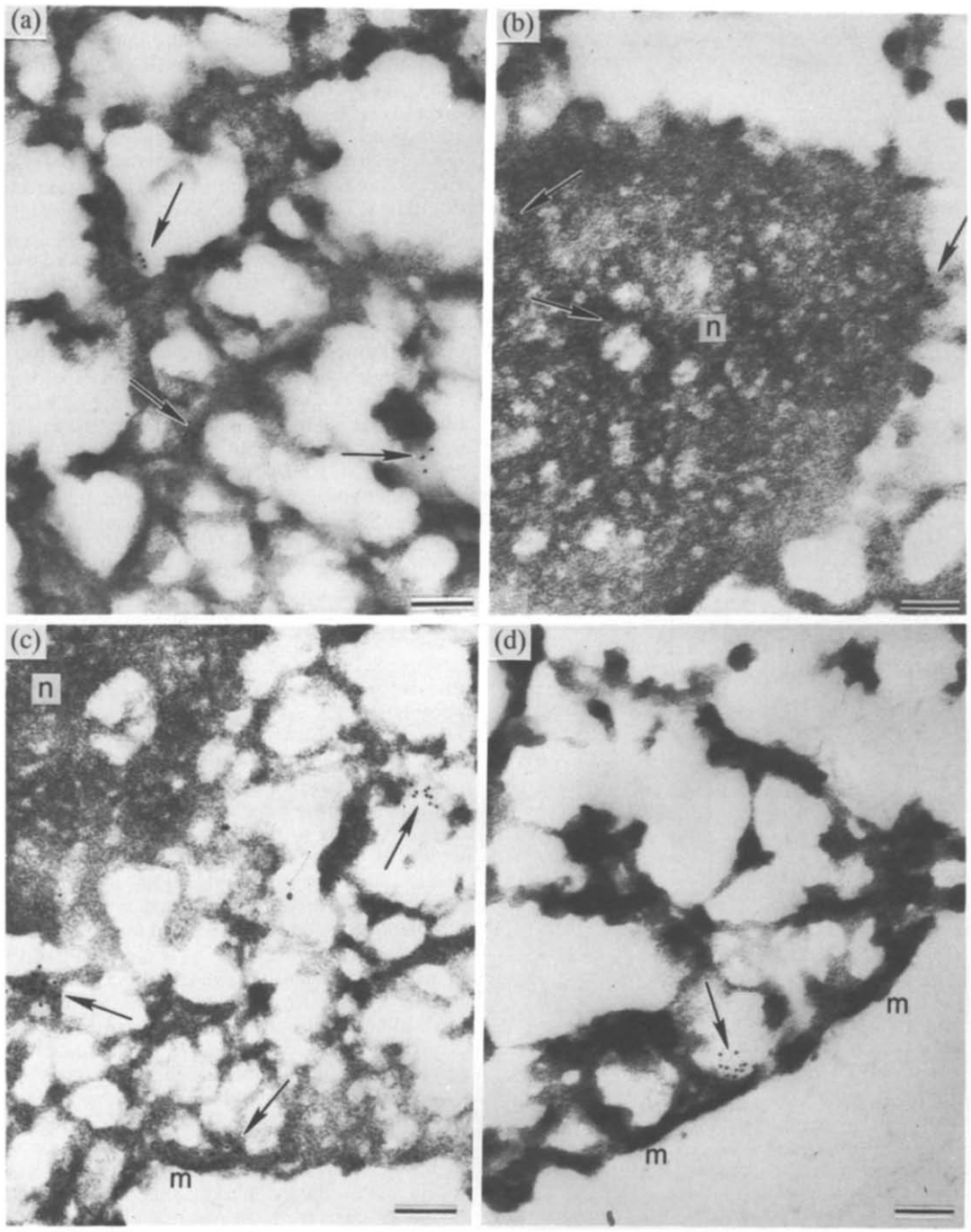

Fig. 5. Electron micrographs of nuclei isolated from HN5 cells which had been treated with EGF (as detailed in the Materials and Methods). Immuno-gold labelling to show the distribution of phosphotyrosine associated with chromatin (arrows in a) and with the nucleolus (arrows in b) and EGF-R (arrows in c and d) immunoreactivity. Bar = 167 nm. The nucleolus (n) and inner nuclear membrane (m) are indicated. No labelling was observed outside of the inner nuclear membrane.

of A431 cells treated with EGF may be because their cells were used after serum starvation for 18 hr prior to application of EGF. Serum starvation has been demonstrated to reduce nuclear transport and import of large proteins; this can be reversed by growth factor treatment but takes >6 hr for recovery to occur [32]. Therefore, regulation of protein import into the nucleus may have prevented access of EGF-R to the nuclear compartment in serum starved cells. In another study by Carpentier *et al.* [33], several

Table 1. Effect of EGF on the amount of EGF-R and phosphotyrosine associated with individual nuclei

	Gold particles per nucleus	
	no EGF	+EGF
EGF-R	28 ± 9	310 ± 35
Phosphotyrosine	49 ± 10	207 ± 22

Isolated nuclei were subjected to immuno-electron microscopy using anti EGF-R or anti phosphotyrosine antibodies as described in the Materials and Methods. For each treatment, the amount of EGF-R and phosphotyrosine associated with randomly selected, intact nuclei was assessed by counting the gold particles of the immuno-conjugate. The results shown are means ± SEM for 20 nuclei in each case.

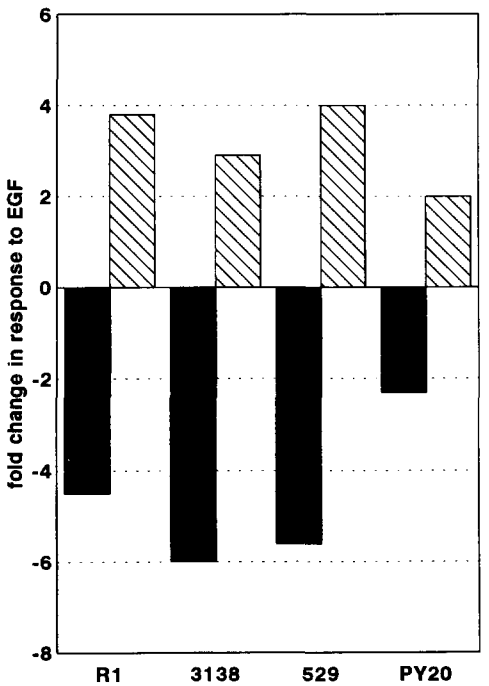

Fig. 6. HN5 cells were incubated in the absence or presence of EGF (100 ng/mL) for 1 hr. Cells were then fixed and processed for immuno-electron microscopy as described in the Materials and Methods. Immunogold particles detecting EGF-R (using antibodies EGF-R1, 3138 and 529 as described in the Materials and Methods) or phosphotyrosine (using antibody PY20) at the plasma membrane (solid bars) or in the nucleus (hatched bars) were counted from 100 randomly selected cells, and changes in labelling expressed as the ratio of values obtained in untreated and treated cells. Negative values indicate a reduction in labelling and positive values indicate an increase in labelling upon EGF treatment.

EGF-R antibodies were used to examine EGF-R distribution in untreated A431 cells; here significant labelling of the nuclear membrane was observed. In our studies where serum containing medium was present throughout, we observed that EGF treatment caused not only labelling of the inner face of the inner nuclear membrane, but also localization of immunoreactivity in the chromatin which was suggestive of 'hot spots' of activity which may correspond to specific regulatory elements in the DNA. This would be consistent with reports that EGF-R binds to chromatin [10]. The translocation of EGF-R to nuclei is even more significant considering several recent reports that two ligands for this receptor, namely amphiregulin and Schwannoma-derived growth factor, are also reported to be translocated from the plasma membrane to the nucleus [34, 35]. In the latter case, nuclear localization was required for mitogenic activity.

Our quantitative immuno-electron microscope study clearly demonstrates the presence of EGF-R, not only in isolated nuclei, but also in nuclei of intact cells treated with EGF. Moreover, nuclear localization of EGF-R could be demonstrated using three antibodies directed against different epitopes in either the extracellular or cytoplasmic domains of the EGF-R. We estimate that between 1 and 10% of total cellular EGF-R can be found in nuclei of EGF treated cells depending on the method of detection used (the ELISA gave lower values than those determined by immuno-EM; this may be because SH2 domain interactions of components of the second messenger signalling pathway with phosphorylated EGF-R hindered access of the second antibody). Our finding of EGF-R in a highly pure, intact nuclear preparation and also in whole cells, poses many more questions than it answers, particularly how a large membrane bound molecule can gain access to the nuclear matrix. Indeed, the finding of protein kinase C and the enzymes of the phosphoinositide pathway [31] in the nucleus is equally perplexing and may indicate that enzymes which function at the plasma membrane have parallel functions in the nucleus. Our experiments do not allow us to distinguish between a direct translocation of EGF-R from the plasma membrane to the nucleus via the endosomes or a process which involves EGF-R present in the cytoplasm. Approximately 5% of intracellular EGF-Rs have been shown to be in the cytoplasm and apparently not membrane associated [33]. By analogy with the IFNα and IFNγ regulated transcription factors, tyrosine phosphorylation of these cytosolic EGF-Rs may induce their translocation from the cytoplasm to the nucleus. Access could be achieved via the nuclear pore and may be mediated by the putative nuclear targetting sequence (**RRR**HIV**RKR**TL**RR**) [30] contained in residues 645–657 of EGF-R. If the translocation involved membrane associated EGF-R, this could again be facilitated by the nuclear targetting sequence (which lies close to the cytoplasmic face of the plasma membrane) and may also require the additional co-operation of nuclear pore associated proteins to act as chaperones. Alternatively, the endosomal membrane containing EGF-R may fuse directly with the nuclear membrane. These possibilities remain to be investigated.

As is frequently observed with cell lines which overexpress EGF-R, the effect of EGF on growth of HN5 cells is a small stimulation of growth at low

doses of EGF, followed by inhibition at higher EGF concentrations [24]. As the concentrations of EGF used in the present study were growth inhibitory, the possibility cannot be excluded that the observed nuclear changes relate to this growth inhibition. However, the changes in nuclear phosphotyrosine and EGF-R content were dose dependent and also occurred at concentrations of EGF which are growth stimulatory of HN5 cells. That nuclear EGF-R may have a role in cell division is consistent with the finding that stabilized complexes of EGF–EGF-R on the cell surface are not able to induce DNA synthesis [36]. Experiments using cells with a mutated EGF-R which cannot internalize have been interpreted as showing that plasma membrane signalling by EGF-R is sufficient to generate a mitogenic response [37, 38]. However, as these mutant receptors no longer have tyrosine autophosphorylation sites which serve to interact with SH2 domains of second messenger generating enzymes [39], interpretation of results from these studies is difficult.

The EGF stimulated translocation of EGF-R to the nucleus provides a mechanism whereby gene activation may be regulated by this growth factor. This may be achieved directly by binding of EGF-R to chromatin, or indirectly by tyrosine phosphorylation of other chromatin associated proteins. The presence of phosphotyrosine containing proteins in nucleoli may indicate that these proteins also have a role in RNA processing. The nature of the tyrosine phosphorylated chromatin-associated proteins and their sites of recognition within the DNA may provide new insight into the mechanisms controlling EGF-stimulated cell division.

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REFERENCES

1. Yarden Y and Ullrich A, Growth factor receptor tyrosine kinases. *Annu Rev Biochem* **57**: 443–478, 1988.
2. Pandiella A, Beguinot L, Vicentini LM and Meldolesi J, Transmembrane signalling at the epidermal growth factor receptor. *Trends Pharmacol Sci* **10**: 411–414, 1989.
3. Ullrich A and Schlessinger J, Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**: 203–212, 1990.
4. Carpenter G and Cohen S, Human epidermal growth factor and the proliferation of human fibroblasts. *J Cell Physiol* **88**: 227–238, 1976.
5. Chao MV, Growth factor signalling: where is the specificity? *Cell* **68**: 995–997, 1992.
6. Schindler C, Shuai K, Prezioso VR and Darnell JE, Interferon-dependent tyrosine phosphorylation of a latent transcription factor. *Science* **257**: 809–813, 1992.
7. Shuai K, Schindler C, Prezioso VR and Darnell JE, Activation of transcription by interferon-γ: tyrosine phosphorylation of a 91 kDa DNA binding protein. *Science* **258**: 1808–1812, 1992.
8. Johnson LK, Vlodavsky I, Baxter JD and Gospodarowicz D, Nuclear accumulation of epidermal growth factor in cultured rat pituitary cells. *Nature* **287**: 340–343, 1980.
9. Rakowicz-Szulczynska EM, Rodeck U, Herlyn M and Koprowski H, Chromatin binding of epidermal growth factor, nerve growth factor, and platelet-derived growth factor in cells bearing appropriate receptors. *Proc Natl Acad Sci USA* **83**: 3728–3732, 1986.
10. Rakowicz-Szulczynska EM, Otwiaska D, Rodeck U and Koprowski H, Epidermal growth factor (EGF) and monoclonal antibody to cell EGF surface receptor bind to the same chromatin receptor. *Arch Biochem Biophys* **268**: 456–464, 1989.
11. Jiang L-W and Schindler M, Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of epidermal growth factor (EGF) binding activity in both 3T3-1 and EGF receptor reconstituted NR-6 fibroblasts. *J Cell Biol* **110**: 559–568, 1990.
12. Marti U, Burwen SJ, Wells A, Barker M, Huling S, Feren AM and Jones AL, Localization of epidermal growth factor receptor in hepatocyte nuclei. *Hepatology* **13**: 15–20, 1991.
13. Podlecki DA, Smith RM, Kao M, Tsai P, Hueckstedt T, Brandenburg D, Lasher RS, Jarett L and Olefsky JM, Nuclear translocation of the insulin receptor. *J Biol Chem* **262**: 3362–3368, 1987.
14. Curtis BM, Widmer MB, de Roos P, and Qwarnstrom EE, IL-1 and its receptor are translocated to the nucleus. *J Immunol* **144**: 1295–1303, 1990.
15. Baldin V, Roman A-M, Bosc-Bierne I, Amalric F and Bouche G, Translocation of bFGF to the nucleus is G_1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J.* **9**: 1511–1517, 1990.
16. Cowley GP, Smith JA and Gusterson BA, Increased EGF receptors on human squamous carcinoma cell lines. *Br J Cancer* **53**: 223–229, 1986.
17. Waterfield MD, Mayes ELV, Stroobant P, Bennett PLP, Young S, Goodfellow PN, Banting GS and Ozanne B, A monoclonal antibody to the human epidermal growth factor receptor. *J Cell Biochem* **20**: 149–161, 1982.
18. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L and Waterfield MD, Expression of epidermal growth factor receptors on human cervical, ovarian and vulval carcinomas. *Cancer Res* **46**: 285–292, 1986.
19. Laemmli UK, Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
20. Armbruster BL, Carleman E, Chiovettei R, Garavito RM, Hobot JA, Kellenberger E and Villinger W, Specimen preparation for electron microscopy using low temperature embedding resins. *J Microsc* **126**: 77–85, 1982.
21. Ericcson JLE and Trump BF, Observations on the application to electron microscopy of the lead phosphate technique for the demonstration of acid phosphatase. *Histochemistry* **4**: 470–485, 1965.
22. Avruch J and Wallach DFH, Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim Biophys Acta* **233**: 334–347, 1971.
23. Carpentier J-L, White MF, Orci L and Kahn RC, Direct visualization of the phosphorylated epidermal growth factor receptor during its internalization in A431 cells. *J Cell Biol* **21**: 2751–2762, 1987.
24. Richter A, Holt SJ, Solic N, Alexander P and Davies DE, Comparison of the mechanisms of growth inhibition of HN5 cells by EGF and anti-EGF-receptor antibody. *Br J Cancer*, submitted.
25. Meek DW and Street AJ, Nuclear protein phosphorylation and growth control. *Biochem J* **287**: 1–15, 1992.
26. Hunter T and Karin M, The regulation of transcription by phosphorylation. *Cell* **70**: 375–387, 1992.
27. Hao Q-L, Ferris DK, White G, Heisterkamp N and Groffen J, Nuclear and cytoplasmic location of the

FER tyrosine kinase. *Mol Cell Biol* **11**: 1180–1183, 1991.
28. Van Etten RA, Jackson P and Baltimore D, The mouse type IV v-abl gene product is a nuclear protein and activation of transforming ability is associated with cytoplasmic location. *Cell* **58**: 669–678, 1989.
29. Kipreos ET and Wang JTJ, Cell cycle-regulated binding of c-abl tyrosine kinase to DNA. *Science* **256**: 382–385, 1992.
30. Laduron PM, Genomic pharmacology: more intracellular sites for drug action. *Biochem Pharmacol* **44**: 1233–1242, 1992.
31. Divecha N, Banfic H and Irvine RF, The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-1) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. *EMBO J* **10**: 3207–3214, 1991.
32. Feldherr CM and Akin D, Regulation of nuclear transport in proliferating and quiescent cells. *Exp Cell Res* **205**: 179–186, 1992.
33. Carpentier J-L, Rees AR, Gregoriou M, Kris R, Schlessinger J and Orci L, Subcellular distribution of the external and internal domains of the EGF receptor in A-431 cells. *Exp Cell Res* **166**: 312–316, 1986.
34. Modrell B, McDonald VL and Shoyab M, The interaction of amphiregulin with nuclei and putative nuclear localization sequence binding proteins. *Growth Factors* **7**: 305–314, 1992.
35. Kimura H, Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity. *Proc Natl Acad Sci USA* **90**: 2165–2169, 1993.
36. Wakshull EM and Wharton W, Stabilized complexes of epidermal growth factor and its receptor on the cell surface stimulate RNA synthesis but not mitogenesis. *Proc Natl Acad Sci USA* **82**: 8513–8517, 1985.
37. Chen WD, Lazar CS, Lund KA, Welsh JB, Chang CP, Walton GM, Der CJ, Wiley HS, Gill GN and Rosenfeld MG, Functional independence of the epidermal growth factor receptor from a domain required for ligand induced internalization and calcium regulation. *Cell* **59**: 33–43, 1989.
38. Wells A, Welsh JB, Lazar CS, Wiley HS, Gill G and Rosenfeld MG, Ligand-induced transformation by a non-internalizing epidermal growth factor receptor. *Science* **247**: 962–964, 1990.
39. Koch CA, Anderson D, Moran MF, Ellis C and Pawson T, SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* **252**: 668–674, 1991.