

Growth factor signalling

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ABSTRACT Signalling between cells in the developing vertebrate embryo is essential for normal embryonic development. In the mid 1970's, signal transduction research started at the Hubrecht Laboratory with special emphasis on analysis of the signalling mechanisms that direct cell proliferation and differentiation. The introduction of *in vitro* model systems contributed tremendously to the success of the signal transduction research at the Hubrecht Laboratory. Initially neuroblastoma cell lines, and later embryonal carcinoma and embryonal stem cells played an important role in identification of the molecular key players in developmental signalling. For instance, embryonal carcinoma cells were used to identify and characterise polypeptide growth factors. Growth factor signalling research was extended to analysis of growth factor receptor activation. Moreover, the second messenger systems that are linked to growth factor receptors were studied, as well as the nuclear responses to growth factor receptor activation. Finally, the role of growth factor signalling in differentiation was established using embryonal carcinoma cells. Here, we will review work that was characteristic for the growth factor receptor signalling research that was done at the Hubrecht Laboratory between 1980 and the early 1990's.

KEY WORDS: *growth factor (receptor), signalling, second messengers, proliferation, development*

Introduction

In the mid 1970's Siegfried de Laat and Paul van der Saag took the initiative to combine their research efforts in forming a new group at the Hubrecht Laboratory, that would focus on the analysis of the molecular nature of the signalling mechanisms guiding cell proliferation and differentiation in vertebrate development. Until then, amphibian embryos were the most widely used objects of study at the Institute, in addition to the mouse embryo and the cellular slime mold *Dictyostelium*. In those days knowledge about the nature of signalling molecules and their receptors was virtually non-existing, but the initiative was nevertheless considered as being promising for the following reasons. First, earlier studies by Pieter Nieuwkoop and others on amphibian embryos had revealed the existence and importance of inductive processes as essential mechanisms in guiding the early differentiation steps in the vertebrate embryo, and contributing to the characterisation of the molecular nature of such processes would thus be a self-evident challenge for our Institute. Second, one could assume on the basis of these studies that the inductive signals would be secreted molecules, which would exert their action by interacting with cell surface components expressed by the responding cells, and

crucial for establishing their developmental competence to respond to such signals. Third, it was felt that the developmental processes taking place simultaneously in the early embryo were of such complexity and diversity, that the introduction and use of simple *in vitro* models was absolutely essential for such an endeavour at that time. Fourth, studies on mammalian cell lines had shown that cells in general had a restricted susceptibility or competence during their cell cycle, i.e. during their G1-phase, for differentiation

Abbreviations used in this paper: EC, embryonal carcinoma; ES, embryonal stem; R(PTK), (Receptor) Protein-Tyrosine Kinase; NRK, normal rat kidney; TGF, transforming growth factor; EGF, epidermal growth factor; PDGF, platelet derived growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; EFF, exoplasmic fracture face; FRET, fluorescence resonance energy transfer; FLIM, fluorescence life-time imaging; DAG, diacylglycerol; AA, arachidonic acid; [Ca²⁺], intracellular free calcium concentration; PKC, protein kinase C; GPCR, G-protein coupled receptor; PLC-gamma, phospholipase C-gamma; SH2, Src homology-2; PLA₂, phospholipase A₂; MAPK, mitogen activated protein kinase; AP1, activator protein-1; TRE, TPA response element; RA, retinoic acid; P.Tyr, phosphotyrosine; PTB, P.Tyr-binding; PI-3 kinase, phosphatidylinositol 3 kinase; GEF, guanine nucleotide exchange factor; Sos, son of sevenless.

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inducing signals. Studying cell cycle related modifications of plasma membrane properties could thus provide clues to understanding the nature of the susceptibility controlling mechanisms. Taken together, these considerations led to the far reaching, and at that time rather controversial, decision to introduce mammalian cell lines as *in vitro* model systems for studying the developmental decision processes in molecular detail by this newly established research group.

Initially, this group focussed on the regulation of growth and differentiation of neuroblastoma and other cell lines, that can be considered as relevant models for neural crest cell differentiation (for a review, see: de Laat and Van der Saag, 1982). With the rapidly expanding knowledge of growth factors and their receptors in the early 1980's, the notion that these constituted ideal candidates as molecular key players in developmental signalling guided much of the further research in this area at the Hubrecht Laboratory. In addition, the availability of murine embryonal carcinoma (EC) cell lines, and subsequently embryonal stem (ES) cell lines as models for studying early differentiation processes in the mammalian embryo had probably an even more important impact on the research programmes of this group and of new groups sprouting from within the Hubrecht Laboratory and elsewhere in the Netherlands in the years to come.

It is beyond the scope of this issue to review all the work carried out at the Hubrecht Laboratory in this field over that many years. For that reason we restrict ourselves here to an overview of some of the signal transduction work characteristic for the period 1980 to the early 1990's, starting with the identification of growth factor activities in various cell lines, and followed by the analysis of the signal transduction mechanisms utilised by Receptor Protein-Tyrosine Kinases (RPTKs), in particular the Epidermal Growth Factor Receptor.

Transforming growth factors

In the late seventies it became clear from the work of De Larco and Todaro that tumour cells were able to secrete growth factors to which they themselves responded in an autocrine manner (De Larco and Todaro, 1978). This revolutionary concept has played a crucial role in our understanding of proliferation processes during embryonic development and tumour growth ever since. Based on studies using virally-transformed murine fibroblasts it was initially believed that these growth factors were encoded by the viral genome, resulting in their first name: "sarcoma growth factor". When it became clear that non-virally transformed cells also produced such factors, and it turned out that these factors were able to induce a transformed phenotype in normal rat kidney (NRK) fibroblasts, they were renamed transforming growth factors (TGFs). TGFs appeared to compete with epidermal growth factor (EGF) for receptor binding, but unlike TGF, EGF was unable to induce a transformed phenotype in NRK cells, suggesting that tumour-derived growth factors had properties that were different from "normal" growth factors. Later studies by Sporn and coworkers indicated that the TGF preparations that were used consisted of a mixture of two growth factors, the EGF-related factor TGF-alpha and a new growth factor, TGF-beta (Anzano *et al.*, 1982). In this way the prototype for the large TGF-beta superfamily was discovered, which turned out to play a vital role in the control of growth, differentiation and embryogenesis.

Cellular responses to transforming growth factors

In a combined study by van Zoelen and Todaro, it was demonstrated that mouse Neuro-2A cells were also able to secrete a transforming growth factor for NRK cells, but in this case no EGFR competing activity appeared to be present (Van Zoelen *et al.*, 1984). Later studies in collaboration with Heldin indicated that besides TGF-beta, these Neuro-2A cells secreted the AA-isoform of platelet-derived growth factor (PDGF). This prompted us to investigate in detail which combinations of growth factors were required to induce loss of density-dependent growth inhibition and anchorage-independent growth of NRK cells, two aspects of phenotypic transformation of these cells. We and others demonstrated that NRK cells had only very low levels of EGFRs, and that the number of EGFRs decreased with increasing cell density. As a result, above a certain critical cell density, EGF was unable to generate sufficient intracellular signals to induce cell proliferation and consequently cells became quiescent even though EGF was present. These cells could be restimulated to proliferate by addition of factors such as TGF-beta, which by itself was not mitogenic for these cells, but enhanced the number of EGFRs, making these cells responsive again to growth stimulation by EGF. Alternatively, other growth factors, such as PDGF, induced proliferation of NRK cells above the critical cell density by activation of signals parallel to those induced by EGF (Van Zoelen, 1991).

Analysis of growth factor expression

As part of the studies to identify growth factor activity secreted by tumour cells, various bio-assays were set up, including assays for detection of EGF, TGF-beta, PDGF, FGF (fibroblast growth factor) and IGF (insulin-like growth factor) activity (Van Zoelen,

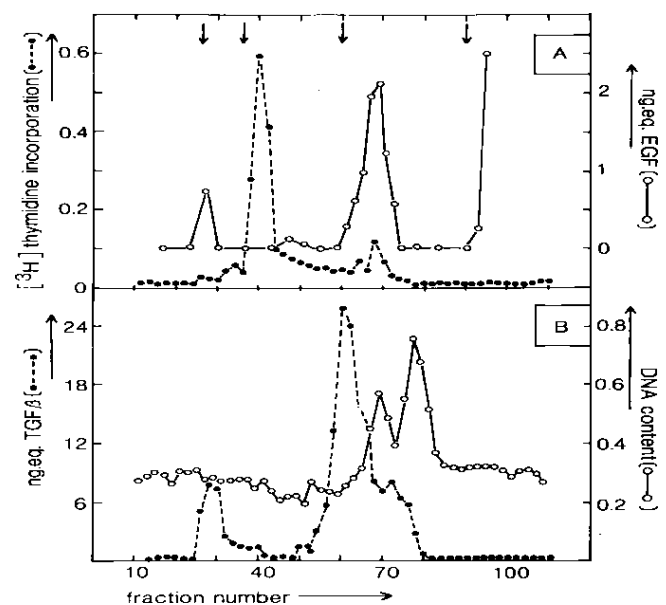


Fig. 1. Elution profile of polypeptide growth factor activity secreted by P19 mouse embryonal carcinoma cells. Fractions were assayed for PDGF-like (A; dashed), EGF-like (A; solid), TGF-beta-like (B; dashed), and IGF-like (B; solid) activities. Arrows in the upper part of panel A reflect the elution position of proteins used for column calibration.

1990). These assays were subsequently used to identify growth factor activities secreted by murine EC cells in culture. Serum-free media conditioned by these cells were lyophilised, extracted with acetic acid and run on a BioGel P-100 column for size separation. As shown by the elution pattern in Figure 1, P19EC cells secreted EGF, TGF-beta, PDGF and IGF-like activities (Van Zoelen *et al.*, 1989). PC13EC cells secreted the same growth factor activities as P19 EC cells as well as a heparin-binding growth factor with FGF-like activity. Techniques for analysing growth factor expression have been strongly improved over the last ten years. For instance, the current PCR and *in situ* hybridisation technologies make it now possible to study expression of growth factors and their receptors directly in embryos. However, bioassays for growth factor activity are still widely used, because these bioassays are very sensitive, and thus enable detection of very low concentrations of growth factor activity. Moreover, these bioassays are generally sensitive to multiple factors within specific growth factor families, and can therefore be used as a first screen to identify the presence of a member of a specific growth factor family.

Epidermal growth factor receptor activation

EGF is structurally and functionally related to TGF-alpha and was first described by Cohen (1962) as a constituent of extracts of submaxillary glands. It was given its name because, when injected into newborn mice, it induced precocious eye-lid opening and incisor eruption, due to growth stimulation of epidermal cells and enhancement of keratinisation. In the seventies, a specific receptor for EGF was identified biochemically (Das *et al.*, 1977) and in 1984 the human EGFR was cloned and the complete primary sequence was deduced (Downward *et al.*, 1984), making the EGFR one of the first receptor molecules for which this was achieved. The receptor turned out to consist of one polypeptide chain of 1186 amino acids which crosses the plasma membrane once. It is heavily glycosylated in the extracellular domain, and contains an intrinsic protein-tyrosine kinase (PTK) activity in its intracellular domain which becomes strongly activated upon EGF binding, resulting a.o. in receptor autophosphorylation on tyrosine residues.

Epidermal growth factor binding to its receptor

Binding studies indicated that the EGFR, like many other growth factor receptors, existed in two affinity classes on the surface of the cell (Shoyab *et al.*, 1979). The high affinity class, occupying about 5-10% of the total population, bound EGF with a K_d of about 10^{-10} M, while the K_d for the low affinity class was about 10^{-9} M. Transfection of full length EGFR cDNA into EGFR free cells resulted in the expression of both affinity classes, showing that the affinity differences must have been caused by posttranslational modifications of the EGFR (Downward *et al.*, 1984). It was unclear whether these modifications were induced by binding of EGF or whether they pre-existed in the unbound state.

During the eighties many research efforts were aimed at elucidation of the molecular mechanism by which binding of EGF to the extracellular domain of the receptor resulted in activation of the

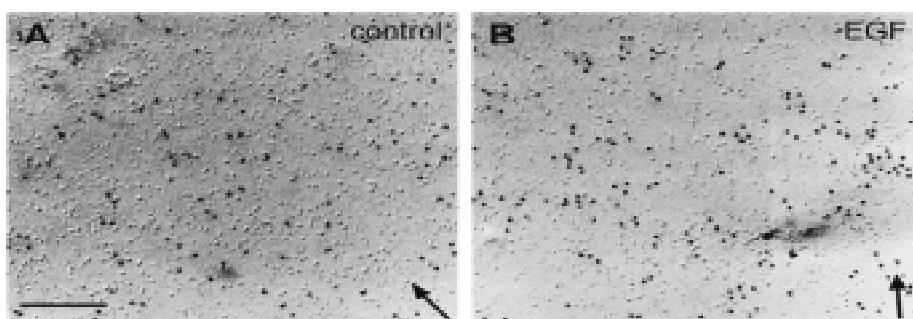


Fig. 2. Rapid EGF-induced receptor clustering. Human A431 cells were immunogold labelled, using anti-EGFR MAb 2E9, and freeze-fractured. Representative electron micrographs of the exoplasmic fracture face of control (A), and EGF-treated, 5 min (B) cells are depicted. Bar, 200 nm, and the arrows indicate the direction of shadowing.

intracellular PTK domain, and what the significance of the two affinity classes and their relative contribution to transduction of the signal was. An important tool in those studies were monoclonal antibodies generated against the receptor. Schreiber *et al.* (1983) were the first to describe such antibodies, and three anti-EGFR monoclonal antibodies were generated at the Hubrecht Laboratory (reviewed by Defize *et al.*, 1987). One of the Hubrecht Laboratory antibodies, 2E9, had the interesting property of competing with EGF for binding to low affinity receptors, but not high affinity receptors, showing that the two affinity states existed even before EGF binding. Using 2E9, we showed that activation of the high affinity subclass of receptors alone was sufficient to evoke not only full receptor activation as measured by autophosphorylation on tyrosine residues, but also the cascade of subsequent intracellular events, finally culminating in cell proliferation (Defize *et al.*, 1989). These results were corroborated by Bellot *et al.* (1990), using monoclonal antibody 108 which specifically competes with EGF-binding to high affinity sites. Interestingly, we also found that the high affinity subclass was present entirely in the Triton X-100 insoluble fraction of cells which was functionally defined as the cytoskeleton, suggesting that receptor-cytoskeleton interactions were important in receptor signalling and binding characteristics (Wiegant *et al.*, 1986). Nevertheless, part of the low affinity subclass was shown to be present in this fraction too, indicating that the receptor-cytoskeleton interaction was not sufficient to generate high affinity binding (Van Bergen en Henegouwen *et al.*, 1989). Indeed, as was shown recently, ablation of an actin binding domain in the receptor did not lead to the disappearance of high affinity sites (van der Heyden *et al.*, 1997). At present, the mechanisms involved in determining ligand affinity of the EGFR are still unclear, although recently it was shown that expression of a mutant form of Dynamin, a molecule involved in directing the EGFR to coated pits, led to the disappearance of high affinity sites, as well as to a strong reduction in ligand-induced autophosphorylation on tyrosine residues (Ringerike *et al.*, 1998). No direct binding of dynamin to the receptor was detected, suggesting that dynamin sequesters another protein involved in modulating the receptors affinity.

Epidermal growth factor receptor activation

With respect to receptor activation, two opposing models existed for a while: the intra-molecular and the inter-molecular model. In the latter model, as was strongly advocated mainly by Schlessinger (1988, 1990), the activation occurred via EGF-

induced receptor dimerisation and thus required at least two receptor molecules. According to the intra-molecular activation model, EGF binding activated the receptor kinase by a conformational change that originated at the external domain and was transmitted to the cytoplasmic domain via the transmembrane stretch within a single receptor molecule (Bertics and Gill, 1985; Gill *et al.*, 1987). In support of this latter model, Weber *et al.* (1984) observed a linear relationship between EGFR concentration and autophosphorylation. Additional evidence was obtained by Koland and Cerione (1988), who demonstrated EGF-induced kinase activity in sucrose gradient fractions containing EGFR monomers only. Since this last publication, no additional results supporting the intramolecular model have been reported.

In the intermolecular activation model, dimerisation, brought about by EGF binding, is sufficient for receptor activation. This model had its roots in the observation of EGF-induced microclustering by immunofluorescence studies (Haigler *et al.*, 1978; Schreiber *et al.*, 1983). Also other evidence indicated that the lateral distribution of the EGFR played an important role in RPTK activation. For example, crosslinking of the EGFRs by antibodies or lectins stimulated receptor autophosphorylation, while immobilisation of the EGFRs on solid matrices prevented activation of receptor kinase activity (Yarden and Schlessinger, 1987a,b). Thus, in this model the activation was not determined by the nature of the ligand *per se*, and the transmembrane domain of the receptor played only a passive role. This model was supported by the observations that neither extension nor shortening of the transmembrane domain by 3 amino acids, nor substitution of hydrophobic amino acids by charged ones resulted in loss of EGF-induced kinase activation (Kashles *et al.*, 1988). Using chemical cross-linkers it was shown that EGF-binding indeed rapidly led to an

increased formation of receptor dimers (Fanger *et al.*, 1986). Furthermore, Yarden and Schlessinger (1987a) demonstrated a parabolic relationship between EGFR concentration and autophosphorylation. To account for EGF-induced dimerisation, these authors subsequently presented a model in which the high affinity sites were present as preformed dimers. EGF would thus preferably bind to dimers, shifting the equilibrium from inactive receptor monomers to active receptor dimers (Yarden and Schlessinger, 1987b).

Epidermal growth factor receptor oligomerisation

A number of electron microscopical methods were developed by many different groups, including ourselves in collaboration with the electron microscopy department of the University of Utrecht. For instance, immunogold labelling techniques were developed, allowing quantitative analysis of the lateral distribution of cell surface located proteins on an ultrastructural level (Pinto da Silva and Kan, 1984; Boonstra *et al.*, 1985). The most simple method for routine use appeared to be the label fracture method developed by Pinto da Silva and Kan (1984). According to this method, cells were immunogold labelled, frozen and subjected to freeze fracture. In contrast to classical freeze fracture, following replication of the fracture faces, the replicas were washed using distilled water without removing the biological material. The gold particles remained attached to the replica and allowed observation of the surface label in projection with the exoplasmic fracture face (EFF) of the plasma membrane in one coincident image, thus permitting analysis of the lateral distribution of the gold particles. Using this method we labelled the EGFRs with the anti-EGFR antibody 2E9 and 10 nm gold particles. We demonstrated that the 2E9 monoclonal antibody bound to the EGFR in a 1:1 ratio (Defize *et al.*, 1989). In the absence of EGF, EGFRs located on the surface of A431 cells were clustered, as deduced from Poisson variance analysis ($p < 0.001$). Following treatment of the cells with EGF at 37°C a rapid increase in EGFR clustering was observed, reaching a maximum within 10 min (Fig. 2). Maximal clustering was maintained for 1 h, after which the lateral distribution of receptors returned to the control situation within another hour (van Belzen *et al.*, 1988).

EGFR activation by dimerisation

The approach, described above, using electron microscopy to investigate the molecular mechanism of RPTK activation was rather unique, since most efforts were aiming at a biochemical approach. For example, in this period it was demonstrated that addition of EGF to purified EGFR preparations as well as to intact cells led to rapid dimer formation (Yarden and Schlessinger, 1987a; Cochet *et al.*, 1988). Others reported that EGFR dimers isolated from purified receptor preparations by sucrose gradient centrifugation displayed a fourfold higher autophosphorylation activity than monomers, while EGF addition shifted the monomer-dimer equilibrium to the dimeric form (Boni-Schetzler and Pilch, 1987). A very important finding strongly supporting the intermolecular activation model was the demonstration that EGFR autophosphorylation was actually mediated by cross-phosphorylation of one receptor molecule by another (Honegger *et al.*, 1987).

Using ^{125}I -EGF we demonstrated that EGF caused an increase in EGFR dimerisation in intact cells after cross-linking, using the bivalent cross-linking reagent disuccinimidyl suberate. Using 2E9

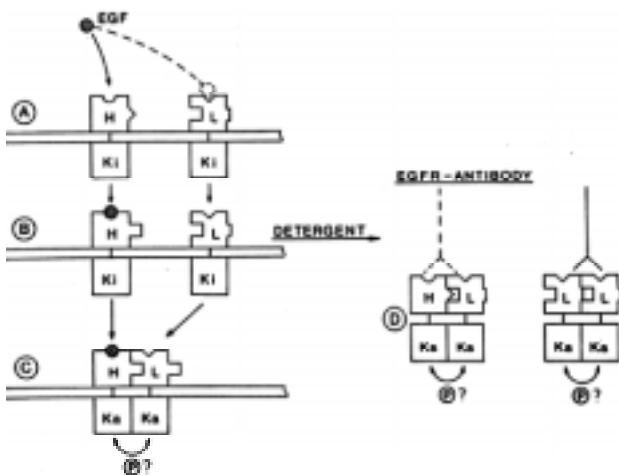


Fig. 3. EGFR activation in intact cells. EGF binding to monomeric high affinity (H) EGFR (A) may facilitate (hetero) dimerisation of high and low affinity (L) EGFR (B), which may be potentiated by a conformational change (indicated in the extracellular domain). Dimerisation brings the cytoplasmic protein-tyrosine kinase domains (Ki, inactive; Ka, active) in close proximity of each other, leading to phosphorylation (P) in trans (C). Bivalent antibodies somehow cannot induce dimerisation in intact cells. Following treatment of these cells with a mild detergent, anti-EGFR antibodies do induce activation of the EGFR (D), thereby bolstering the hypothesis that dimerisation induces activation of the EGFR.

as a discriminating antibody between the high and low affinity class receptors we further demonstrated that the high affinity receptors consisted of both monomers and dimers. Therefore, these findings contradicted the high affinity = dimer model.

Another important prediction of the active dimer hypothesis was that the activation of the EGFR should not be strictly confined to the natural ligand. In essence, all ligands that were able to dimerise the receptor should in principle also have been able to activate the RPTK. In this respect it was of interest that bivalent antibodies were able to induce EGFR autophosphorylation and PTK activation in *in vitro* phosphorylation assays (Gill *et al.*, 1984; Defize *et al.*, 1986; Yarden and Schlessinger, 1987a). However, despite their bivalent nature, antibodies were not able to activate the EGFR PTK in intact cells (Gill *et al.*, 1984; Defize *et al.*, 1989). Therefore, we studied this phenomenon in more detail. Using antibodies directed against the external or internal domain of the EGFR, we were able to activate the EGFR PTK both in membrane preparations as well as in intact cells. However, this activation was only observed if the membranes or cells were treated with a mild detergent (Fig. 3) (Defize, 1988; Spaargaren *et al.*, 1990). In agreement with the active dimer model, the activation of the RPTK was observed irrespective of the recognition site of the antibody (Spaargaren *et al.*, 1990). Subsequently, we demonstrated –using similar protocols– that the activation of the receptor kinase by antibodies was accompanied by receptor dimerisation as well, again depending on the presence of detergent (Spaargaren *et al.*, 1991). If the antibody bivalency was changed to monovalency by the preparation of Fab fragments, no activation and no dimerisation was observed. Subsequent addition of a bivalent secondary antibody restored both the activation and the dimerisation (Fig. 3) (Defize, 1988; Spaargaren *et al.*, 1991). Interestingly, partial inhibition of the PTK activity by tyrphostin inhibited autophosphorylation, but not dimerisation of the EGFR. Taken together, these data indicated that EGFR dimerisation was essential for kinase activation, but not *vice versa*.

Currently, the exact mechanism of receptor activation is still unclear, as is the exact role of the high and low affinity receptor populations. One of the latest findings, supporting the intermolecular activation model was obtained through the use of the recently developed fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) techniques. Using FRET, interactions between individual fluorescently labelled molecules can be monitored with high precision and sensitivity, while using FLIM, the behaviour of one single labelled receptor molecule can be followed. Based on FRET and FLIM experiments, Jovin and co-workers hypothesized that binding of EGF caused a rotational change in two closely apposed receptors (preformed dimers), such that the intracellular kinase domains now interacted and cross-phosphorylated each other (Gadella and Jovin, 1995). Development and optimisation of such techniques will in the near future undoubtedly yield additional clues about the activation mechanism of the EGFR.

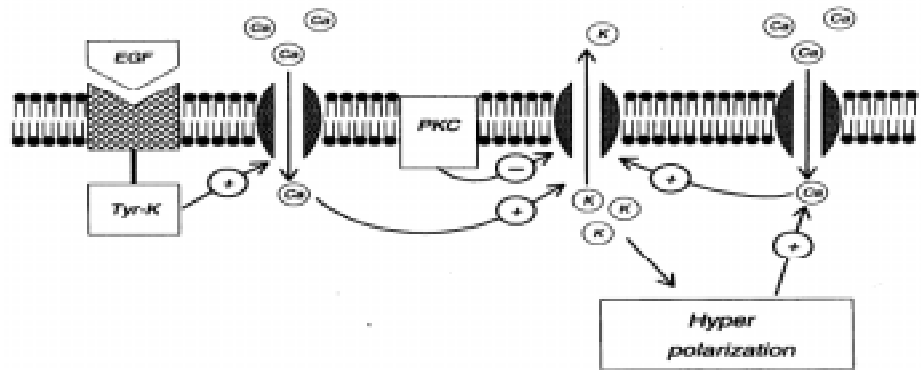


Fig. 4. Model for EGFR activation-induced elevation of the intracellular free Ca²⁺-concentration ([Ca²⁺]_i). EGFR activation induces a small Ca²⁺ influx, which initiates an autocatalytic process by activation of a K⁺-channel. Activation of this K⁺-channel in turn leads to hyperpolarisation of the membrane potential, which is a trigger for hyperpolarisation-dependent Ca²⁺-channel activation. Protein Kinase C (PKC) mediated inhibition of the K⁺-channel leads to attenuation of the EGF-induced rise in [Ca²⁺]_i.

Epidermal growth factor receptor activation: the second messengers

The binding of polypeptide growth factors to their receptors initiates a variety of rapid intracellular changes. As described above, one of the first steps of signal transduction following binding of a polypeptide growth factor to its receptor is tyrosine specific phosphorylation of the receptor itself, shown for the first time by Ushiro and Cohen (1980). Subsequently, activated growth factor receptor PTKs induce the production of a whole cascade of second messenger products, including intracellular free Ca²⁺, inositol phosphates, diacylglycerol (DAG) and arachidonic acid (AA). In fact, PTK activity of growth factor receptors is essential for signalling, since ligand binding to kinase inactive EGFR with a point mutation in the ATP binding site does not lead to an increase in intracellular free Ca²⁺ and pH, nor to production of inositol phosphates (Moolenaar *et al.*, 1988).

Calcium and inositol phosphates

At the end of the seventies, two different groups discovered that growth factors induced rapid alterations in calcium concentration upon growth factor stimulation, using ⁴⁵Ca²⁺ (Tupper, 1978; Sawyer and Cohen, 1981). Using fluorescent intracellular calcium indicators, Quin-2 and - more recently - Indo-1 and fura-2, we started at the Hubrecht Laboratory to study calcium homeostasis in cells directly. In 1984 we found that several polypeptide growth factors, including EGF and platelet derived growth factor (PDGF), rapidly induced changes in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) (Moolenaar *et al.*, 1984). This was the beginning of a series of publications in which the kinetics of changes in [Ca²⁺]_i and the underlying mechanism were reported in increasing detail. We found that a large component of the EGFR-mediated rise in [Ca²⁺]_i was due to influx from the extracellular medium. Moreover, we showed a direct involvement of protein kinase C (PKC) in the regulation of [Ca²⁺]_i, in that brief activation of PKC with the phorbol ester TPA fully inhibited the EGF-induced Ca²⁺-influx (Moolenaar *et al.*, 1986). A drawback from the early studies was the use of the calcium indicator Quin-2, which had high calcium buffering capac-

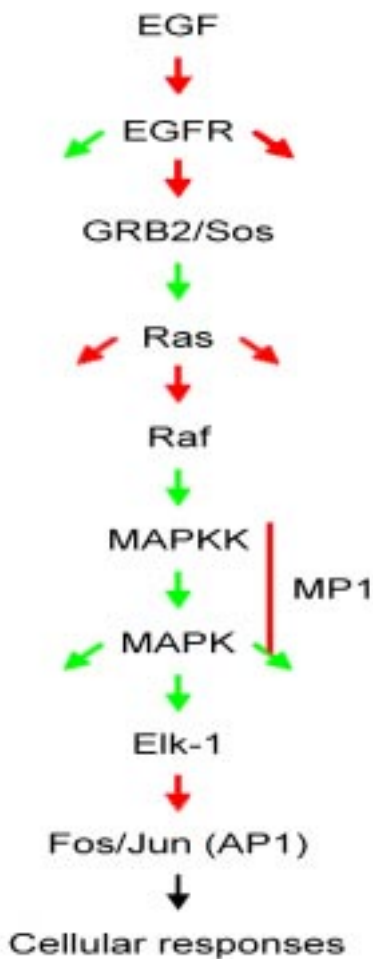


Fig. 5. The RPTK-Ras-MAPK signalling pathway. Red arrows indicate direct protein-protein interactions, green arrows enzyme substrate interactions. See text for details.

ity, thereby masking subtle differences in amplitude and time. The successors of Quin-2, Indo-1 and fura-2, allowed a more detailed view of calcium homeostasis, revealing receptor mediated calcium influx, as well as release of calcium from intracellular stores.

Another important class of second messengers that was affected by EGFR activation is the class of inositol phosphates. In fact, $[Ca^{2+}]_i$ and inositol phosphates were connected, in that inositol phosphates triggered the release of calcium from intracellular stores (Berridge and Irvine, 1989). In 1986 we started at the Hubrecht laboratory with radioactive inositol phosphate measurements by liquid chromatography (HPLC), which allowed separation and quantification of the individual inositol phosphate products and a direct comparison between inositol phosphate production and accompanying changes in $[Ca^{2+}]_i$. Inositol phosphate production and calcium metabolism following treatment with EGF and bradykinin were compared to study differences in signalling by the EGFR, a RPTK, and a G-protein coupled receptor (GPCR), the bradykinin receptor. We found that both receptor classes elicited inositol phosphate production. EGF, however, showed a slow but persistent release of inositol phosphate formation during a period of 30 min, with only a minor rapid, transient increase in inositol

1,4,5-triphosphate. Bradykinin evoked an immediate and transient production of inositol 1,4,5-triphosphate, exactly matching the short and massive rise in $[Ca^{2+}]_i$, which was characteristic for G-protein coupled receptors, and which was entirely due to release of calcium from intracellular stores (Tilly *et al.*, 1988). Our observations fitted in with, and contributed to rapidly emerging knowledge of RPTK signalling at the cell membrane.

As it turned out, EGF-induced inositol phosphate production was mediated directly by Phospholipase C-gamma (PLC-gamma). PLC-gamma is a direct target of activated RPTKs, in that PLC-gamma bound to activated, autophosphorylated RPTKs via its Src Homology 2 (SH2) domain and P.Tyr in autophosphorylated RPTKs. PLC-gamma was then activated by RPTK-mediated tyrosine phosphorylation, leading to the formation of inositol 1,4,5-triphosphate. In addition, activation of PLC-gamma induced production of the second messenger, DAG, a potent activator of several subtypes of PKC. PKC was involved in downregulation of the EGFR PTK activity. In addition, PKC was a key enzyme in signal transduction and was found to play a role in activation of the Na^+/H^+ exchanger in our laboratory and by others (Moolenaar *et al.*, 1983; Sardet *et al.*, 1990), thereby regulating the intracellular pH.

Mechanism of calcium influx

In 1989 it was still far from clear how EGFR activation led to calcium influx. We soon realised that the elegant technique of single channel patch-clamp (Hamil *et al.*, 1981) could provide new and detailed insights into the ion channels involved. In a study in 1991 (Peppelenbosch *et al.*, 1991), we elucidated a mechanism in which EGFR activation triggers an autocatalytic ionic cascade in which voltage-independent activation of Ca^{2+} -channels is the primary response. The resulting Ca^{2+} -influx causes increased activity of Ca^{2+} -dependent K^+ -channels, leading to hyperpolarisation of the membrane potential which in turn results in further Ca^{2+} -influx due to activation of hyperpolarisation-dependent Ca^{2+} -channels. In addition, we found a negative feedback mechanism in which PKC mediates downregulation of K^+ -channel activity, eventually leading to cessation of the ionic response (Fig. 4).

Arachidonic acid

The possible importance of AA as a second messenger of EGFR signalling was demonstrated in two studies. Handler *et al.* (1990) and Sellmayer *et al.* (1991) showed that EGF-evoked induction of c-myc, *egr1* and c-fos expression was blocked by inhibiting the AA metabolism. AA is a product of phospholipase A_2 (PLA₂)-mediated metabolism of phospholipids, suggesting a role for cPLA₂ in long-term EGFR signalling. We demonstrated that cPLA₂ was activated in response to EGF, resulting in the production of AA (Spaargaren *et al.*, 1992). Mitogen Activated Protein Kinase (MAPK), which is activated in response to RPTK activation, phosphorylated and thereby activated an 85 kDa form of cPLA₂ (Lin *et al.*, 1992; 1993). cPLA₂-activation appeared to be an important step in growth factor signalling, since the induction of a number of early response genes was eliminated upon interference with AA metabolism (Handler *et al.*, 1990). Similarly, we found that EGF-dependent induction of the *junB* early response gene expression was sensitive to inhibition of leukotriene synthesis (Peppelenbosch *et al.*, 1992). AA not only played a role in early response gene expression, but also in calcium homeostasis, since we found a direct role for AA-metabolites, especially lipoxygenase products, in the activation of the calcium channels in A431 cells in

response to EGFR activation (Peppelenbosch, 1992). Recently, we found a direct connection between EGF-stimulated phosphorylation and activation of cPLA₂ and translocation to the membrane in a positive feed forward loop, involving Ca²⁺ (Schalkwijk *et al.*, 1995).

Actin reorganisation

It has been known for a long time that RPTK stimulation often initiated pronounced morphological changes, which were driven by reorganisation of the actin cytoskeleton (e.g. Schlessinger and Geiger, 1981; Rijken *et al.*, 1991). Two different mechanisms of regulating the actin microfilament system have been discovered, involving the small Ras-related GTP-binding proteins Rac and Rho (Ridley and Hall, 1992; Ridley *et al.*, 1992). We proposed a regulatory role of AA and its metabolites in these processes (Peppelenbosch *et al.*, 1993). The coupling of the small GTPases Rac and Rho in EGF-evoked AA generation was studied in more detail in 1995 (Peppelenbosch *et al.*, 1995). Whereas lysophosphatidic acid induced stress-fibers solely in a Rho-dependent manner, independent of Rac (Ridley and Hall, 1992; Jalink *et al.*, 1994), EGF-induced cPLA₂ activation and subsequent AA production was found to exert its action in a Rac-dependent way (Peppelenbosch *et al.*, 1995; Kim and Kim, 1997). However, actin remodeling and the subsequent changes in cell morphology is a complex and still ill-understood regulatory mechanism.

Nuclear responses to growth factor receptor activation

The first reports demonstrating that growth factor action involved a nuclear response date back to the early eighties, when it was shown that EGF and PDGF rapidly and transiently induced the expression of the *c-fos* and *c-myc* proto-oncogenes in NIH 3T3 cells (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984). Expression of these so-called "immediate early" genes is regulated at the level of transcription, starting within minutes after growth factor-receptor interaction. In addition, expression of these genes is independent of *de novo* protein synthesis, indicating that all components for receptor-downstream signalling are present in the cells. It soon became apparent that not only growth factors but also a large variety of other extracellular stimuli induces expression of the *c-fos* gene, including receptor agonists that raise intracellular levels of cAMP and cytokines such as TGF- β and interleukins.

Induction of *c-fos* expression represented an attractive system to investigate the nature of receptor-downstream signal transduction leading to gene expression in the nucleus. This approach was complementary to ongoing research at the Hubrecht Laboratory that was mainly focussed on plasma membrane localised ionic and biochemical events (as described above). Investigation in A431 cells showed that besides EGF also TPA-induced activation of PKC or a sustained rise in [Ca²⁺]_i using the Ca²⁺ ionophore A23187 resulted in rapid induction of *c-fos* expression. Both pathways were activated simultaneously through EGF receptor-mediated breakdown of plasma membrane inositol lipids and collectively contributed to the induction of the *c-fos* gene. Furthermore, *c-fos* induction by EGF was found to be dependent on the entry of extracellular Ca²⁺, in line with the effects of extracellular Ca²⁺ on expression of the *junB* gene (Peppelenbosch *et al.*, 1992). By contrast, the induction of *c-fos* expression by bradykinin was completely dependent on the release of Ca²⁺ from intracellular stores.

Given the multitude of factors and complexity of intracellular signal transduction pathways, it soon became clear that studying intracellular signal transduction could better be approached at the level of the *c-fos* promoter-enhancer, as first demonstrated by Treisman (1985). These studies resulted in the identification of multiple response elements and transacting factors such as Elk-1, mediating the transcriptional response of the *c-fos* gene to growth factors (Fig. 5). Furthermore, it was found that Fos readily forms a complex with Jun and the Fos/Jun complex was referred to as Activator Protein 1 (AP1). The AP1 complex has DNA binding activity and interacts with a heptanucleotide sequence referred to as TPA Response Element (TRE) that was identified on the basis of its transactivation potential in response to activation of protein kinase C (Angel *et al.*, 1988). The identification of transcription factors such as Elk-1 and AP1 that regulate the immediate early transcriptional response in response to growth factors has greatly contributed to functional characterisation of the RPTK-Ras-MAPK signal transduction pathway as we know it today.

Jun/AP1 and EC cell differentiation

Initial characterisation of the induction of *c-fos* by TPA in P19 EC cells revealed resistance to TPA in undifferentiated P19 EC cells that was not observed in retinoic acid (RA)-differentiated derivatives. The induction of *c-fos* by TPA in differentiated derivatives of P19 EC cells corresponded with increased expression of EGF receptors on the differentiated derivatives as well as enhanced EGF responsiveness with regard to cell proliferation (Mummery *et al.*, 1985). These results suggested that during P19 EC differentiation both the expression of growth factor receptors as well as components involved in activation of the nuclear response were differentially regulated. To further investigate this issue, the expression and TPA induction of the *jun* and *fos* family of genes was investigated in undifferentiated as well as RA differentiated derivatives of P19 cells (de Groot *et al.*, 1990b). Differentiated P19 cells showed an upregulation of *c-jun* and an increased TRE transactivation due to induction of *fos* and *jun* genes by TPA. The lack of *c-jun* expression in undifferentiated P19 EC cells is functionally significant in maintaining the undifferentiated state of P19 EC cells as ectopic expression of c-Jun leads to differentiation yielding mainly endoderm derivatives (de Groot *et al.*, 1990a). In addition, ectopic expression of the EGF receptor in undifferentiated P19 EC cells results in EGF-induced *c-jun* expression and TRE transactivation (den Hertog *et al.*, 1991, 1992). These results demonstrate that EC cells are competent to respond to growth factors by induction of early response gene transcription, as well as point to c-Jun as an important mediator of cellular differentiation. These findings corroborate more recent data concerning the mechanisms of growth factor signal transduction in EC and ES cells as well as the function of c-Jun in growth control and differentiation.

Current growth factor receptor signalling research

The signal transduction research field evolves rapidly, not in the least part due to rapid development of new, sophisticated techniques to study cell signal transduction at the molecular level. Currently, the work is mostly aimed at elucidating signalling pathways by analysis of direct protein-protein interactions, or direct enzyme-substrate interactions (Hunter, 1997). Analysis of these interactions is often aided by, or even based directly on genetic

pathways in invertebrates. The identification of protein modules that mediate protein-protein interactions was of crucial importance and has provided much insight into possible binding protein/target protein interactions (reviewed by Pawson, 1995). Ligand-binding to a growth factor receptor with intrinsic PTK activity leads to tyrosine phosphorylation of target proteins, most notably itself, due to autophosphorylation *in trans*, as described above (Lemmon and Schlessinger, 1994). These P.Tyr residues in activated RPTKs provide binding sites for proteins that contain SH2 domains, or phosphotyrosine binding (PTB) domains. SH2 domains bind to P.Tyr in specific target proteins, and the specificity is directed by the sequence to the C-terminal side of the P.Tyr (Songyang and Cantley, 1995). Autophosphorylated RPTKs bind many different SH2 and PTB domain-containing proteins, including proteins with intrinsic enzymatic activity, e.g. PLC-gamma (see above), and adaptor proteins without enzymatic activity. Phosphatidylinositol 3 kinase (PI-3 kinase) is an intermediate, since it consists of an adaptor protein, p85, that binds to activated RPTKs and a catalytic subunit, p110, that is constitutively bound to p85 and becomes activated upon binding to activated RPTKs. Shc is a *bona fide* adaptor protein that binds to activated RPTKs through a PTB domain (van der Geer and Pawson, 1995). Upon binding, Shc is phosphorylated on tyrosine and, in turn, provides binding sites for other SH2 domain containing proteins. Recently, many more adaptor proteins have been identified, without intrinsic enzymatic activity, but with multiple binding domains and binding sites. The function of these adaptor proteins may be to form scaffolds for signalling proteins, thereby integrating the different signals (Pawson and Scott, 1997).

The RPTK-Ras-MAPK signalling pathway

One –by now classical– example of a signalling pathway that was elucidated is the RPTK–Ras– MAPK signalling pathway (Fig. 5)(reviewed by Schlessinger, 1993). In fact, this signalling pathway was solved genetically in parallel in *C. elegans* and *Drosophila* (Clark *et al.*, 1992; Olivier *et al.*, 1993). One of the proteins that binds to autophosphorylated RPTKs is the adaptor protein GRB2 (Sem-5 in *C. elegans* and Drk in *Drosophila*) (Lowenstein *et al.*, 1992). GRB2 not only contains an SH2 domain, but also two SH3 domains, flanking the SH2 domain. SH3 domains are protein modules that bind to specific proline rich sequences in target proteins (Pawson and Scott, 1997). The SH3 domains of GRB2, especially the N-terminal SH3 domain binds constitutively to proline-rich sequences in the guanine nucleotide exchange factor (GEF) for the Ras GTPase, Son of Sevenless (Sos) (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Gale *et al.*, 1993). As a consequence, binding of GRB2 to activated RPTKs leads to translocation of Sos to the plasma membrane, where its target is located, Ras. Translocation of Sos to the membrane apparently is sufficient for activation of Sos (Aronheim *et al.*, 1994). Sos mediates GTP-loading and thus activation of Ras. Activated Ras, in turn, has at least three effectors: a Ser/Thr protein kinase, Raf1, a lipid kinase, PI-3 kinase, and a GEF for Ral (another small GTPase), Ralf (reviewed by Bos, 1998). Raf1 is activated by binding to Ras and is upstream of a kinase cascade that includes MAPK-Kinases and MAPKs (ERKs) (Marshall, 1995). Recently, scaffold proteins were identified that may bind multiple members of MAPK cascades (reviewed by Whitmarsh and Davis, 1998). For instance, MP1 specifically binds MAPK-Kinase1 and ERK1 and facilitates their activation (Schaeffer *et al.*, 1998). Downstream

effects of activated MAPK include phosphorylation and activation of transcription factors, including Elk-1 (Yang *et al.*, 1998), which may lead to permanent changes in the target cells through direct transactivation of the *c-fos* gene.

Taken together, one signalling pathway from activated growth factor receptors to the nucleus via direct protein-protein and/or enzyme-substrate interactions has been solved (Fig. 5). However, this is by no means the only signalling pathway that leads to permanent changes in the target cells upon growth factor treatment. As discussed above, many other signalling components are activated upon growth factor receptor activation as well. Moreover, many other factors positively and negatively affect the RPTK-Ras-MAPK signalling pathway. The complexity of RPTK signalling is illustrated, for instance, by the fact that PI-3 kinase is not only activated directly by activated RPTKs, but also by a downstream signalling component, activated Ras.

EGFR signal transduction in development

By now, several signal transduction pathways from the cell membrane to the nucleus have been solved (in part) at the molecular level. Elucidation of the role of these signalling pathways *in vivo* is a challenge. One way to go about this is to knock out the different components of these signalling pathways and study the possible (developmental) defects of mice lacking these components. Experiments along these lines are currently underway, and recently, ablation of several components of the EGFR-Ras-MAPK pathway has been reported. For instance, ablation of the EGFR itself results in various defects, mostly linked to impaired epithelial development. The severity of some aspects of the EGFR knock-out phenotype are strain-dependent (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). EGFR^{-/-} mice die within three weeks after birth and exhibit severe, progressive neurodegeneration which is strain-independent, suggesting that the EGFR plays a role in proliferation and/or differentiation of astrocytes and in the survival of postmitotic neurons (Sibilia *et al.*, 1998).

Not only the EGFR, but also other components of the pathway have recently been knocked out. For instance, ablation of the adaptor protein GRB2 results in major defects during early development (Cheng *et al.*, 1998). GRB2 is required for normal endodermal cell differentiation during embryogenesis and for proper formation of the epiblast. Due to the severity of the phenotype of the GRB2 knock-out, it is impossible to deduce what the function of GRB2 is in EGFR signalling *in vivo*. More sophisticated genetic experiments using conditional knock-outs and/or substitutions with mutant signalling molecules with subtle mutations (e.g. mutations knocking out the GRB2 binding sites in the EGFR) will eventually provide insight into the role of all components in the RPTK-Ras-MAPK signalling pathway, and thus lead to elucidation of the role of RPTK signalling in development *in vivo*.

Concluding remarks

The work on signal transduction at the Hubrecht Laboratory has contributed to our understanding of the early events following growth factor receptor activation. Most notably, we have established that high and low affinity EGFRs exist, that high affinity binding is essential for EGF-induced cellular responses, that the

EGFR is activated by dimerisation, and that EGFR activation leads to changes in second messenger production, and to early response gene expression. These days, most of the signal transduction work is aimed at elucidation of direct protein-protein or enzyme-substrate interactions. However, it is crucial to keep the ionic changes in mind, since even subtle changes in pH or Ca^{2+} concentration may have dramatic effects on enzymatic activities. These days, the effects of RPTK-induced changes in intracellular pH and Ca^{2+} on enzymatic activities of downstream signalling components are often underestimated.

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