The Role of Epidermal Growth Factor Mediated Signaling in Mitogenic Activation of Hepatocytes

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Abstract

Epidermal growth factor (EGF) is important in the regulation of proliferation in hepatocytes. The receptor for EGF belongs to the receptor tyrosine kinase family, and activates different intracellular signaling proteins by tyrosine phosphorylation. The EGF receptor can activate several signaling pathways, and their role in growth regulation has been extensively studied. But the molecular mechanisms that modulate the different signaling pathways are not clear. This study focuses on the regulation of STAT signaling by the EGF receptor, and particularly the involvement of Stat5b in growth control in hepatocytes. We found that the EGF receptor activated Stat5b by phosphorylation on residue Tyr-699 and other sites, but it was the Tyr-699 phosphorylation that correlated with the DNA binding activity. This activation was dependent of the non-receptor tyrosine kinsase Src, but Stat5b activation by other growth factors was not. Furthermore, the EGF induced Stat5b activation was downregulated in mitogenically responsive hepatocytes, and provides more evidence against Stat5b involvement in mitogenic signaling from the EGF receptor in hepatocytes. In addition we examined the mechanism behind the comitogenic effect of G protein coupled receptor (GPCR) ligands on EGF induced growth. Vasopressin, norephenephrine, angiotensin II, and prostaglandin $F_{2\alpha}$ are such ligands, and they acts as comitogens in hepatocytes. We found that in hepatocytes GPCR ligands did not transactivate the EGF receptor, but rather regulated the transcription of early response genes *c-Myc* and *ATF3*.

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Special thanks to Tormod Guren and Laila Nilsen for the cooperation in the making of the papers presented here.

Finally, I am grateful for the opportunity to work with the talented and skillful colleagues at the Department of Pharmacoloy during these years: You are a great gang!

Sincerely

John Ødegård
Oslo March 2004

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The Role of Epidermal Growth Factor Receptor Mediated Signaling in Mitogenic Activation of Hepatocytes.

General purpose of this study

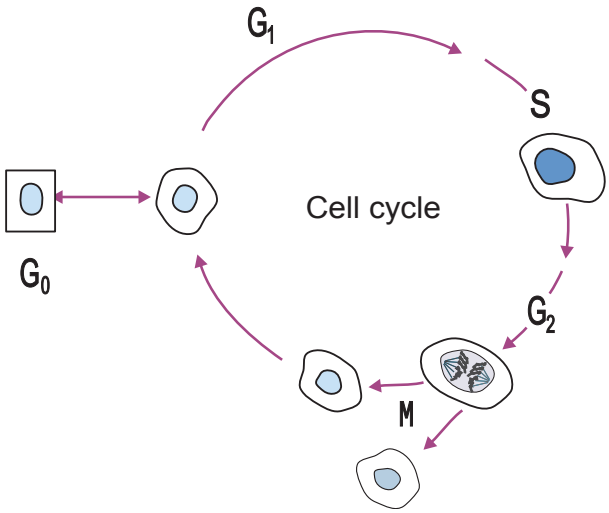
This study concentrates on the activation and intracellular signaling from the EGF receptor in the regulation of cellular proliferation, using hepatocytes in primary cultures as a model. Activated EGF receptor can use various intracellular signaling pathways, but how the receptor regulates its signaling cascades to induce cell growth is unclear. Insight into these molecular mechanisms and pathways is important for understanding normal and pathological cell growth, and may give us potential targets for pharmacological interference. Cancer is a disease where control of cellular signaling is lost, resulting in uncontrolled proliferation.

Introduction

Cell-division cycle

Cells reproduce by dividing into two after duplicating their contents. The sequence of events leading to cell division is called the cell cycle, which consists of different phases (see fig. 1). Most cells in an adult body are in a resting state, the G_0 phase, and are not active dividing. In the G_0 phase the cells are performing specialized functions while they are under strict control mechanisms that inhibit their ability to proliferate (van der Geer et al., 1994; Schlessinger, 2000). To exit the G_0 phase and progress into the cell cycle, cells are dependent upon stimulatory signals (O'Farrell, 1992). Growth factors are peptides that may act as such signals. They can stimulate the

Figure 1: The Cell cycle



cell to advance into the G_1 phase and enter S phase followed by cell division. On the contrary, other peptides can be inhibitory and holdback the advancement in the cell cycle.

The passage through the restriction point and entry into S phase is controlled by cyclin-dependent protein kinases (Cdk), which themselves are regulated by cyclins and Cdk inhibitors. An example is the phosphorylation of the retinoblastoma tumor suppressor protein (RB) by cycline-D1 Cdk's, which in many cell systems is required for entry to S phase (Sherr, 1996).

Growth factors and receptor tyrosine kinases

Growth factors are polypeptides that regulate cellular proliferation, but they may have a number of other effects. They function largely in a paracrine or autocrine fashion, and exert their actions by binding to and activating specific receptors. Activation of receptors is the first step in a series of intracellular events leading to regulation of the cell cycle machinery or other functions (van der Geer et al., 1994).

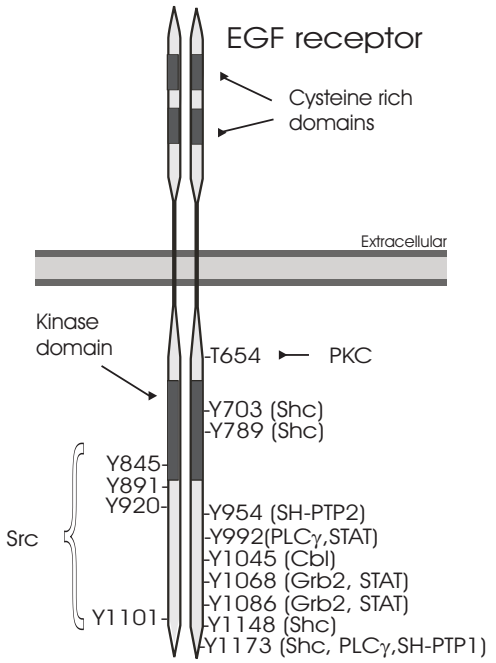
One group of receptors is membrane spanning cell surface receptors, which can be classified by their biological action. A large family is receptors which have intrinsic protein tyrosine kinase activity, so called receptor tyrosine kinases (RTK) (Schlessinger, 2000). In this family we find the receptors for growth factors like epidermal growth factor, which was the first to be discovered, and platlet derived growth factor (PDGF). The EGF receptor has been extensively studied as model for intracellular signaling in the regulation of proliferation. Also in this family is the receptor for insulin, which traditionally is not classified as a growth factor, but has many and complex effects on growth in hepatocytes (Christoffersen et al., 2000).

EGF receptor and agonists

The EGF (EGFR = ErbB1 = HER1) receptor belongs to the receptor tyrosinkinase familiy (RTK) (Carpenter and Cohen, 1990; Schlessinger, 2000). Together with Erb2, Erb3 and Erb4 (HER1 to HER 4) they constitute the HER/ErbB superfamily (Prenzel et al., 2001). The EGF receptor (M_w=170 000) is composed of a single polypeptide chain, containing 1 186 aminoacids, and with approximately 40 kDa N-coupled oligosaccharides extracellularly. It has an extracellular ligand binding domain and an intracellular domain witch contains the tyrosine kinase and the different tyrosine residues

that act as docking sites for signaling proteins when phosphorylated (fig. 2). Between the extra- and intracellular domain is a hydrophobic transmembranal part, and a intracellular juxtamembranal domain (Carpenter and Cohen, 1990).

Figure 2: EGF receptor homodimer. *Cytoplasmic phophorylation sites and dockingsites for intracellular signaling proteins are shown.*



EGF it is a 6.0 kDa (53 aminoacids) heatstable polypeptide, which along with NGF was the first growth factor to be characterized (Savage et al., 1972). Since then a large number of EGF receptor and receptor family ligands have been described (see table 1). Common for all is that they are derived from transmembrane precursors that are cleaved to give rise to soluble ligands. They can be divided into three classes depending on their binding specificities. One group includes EGF itself, transforming growth factor alpha (TGFα), amphiregulin (AR), and epigen (Savage et al., 1972; Beerli and Hynes, 1996; Harris et al., 2003). Each of these ligands can all bind the EGF receptor (ErbB1), and they all compete for binding to the receptor. Another class contains the ligands that bind ErbB4 in addition to the EGF receptor, and here we find the ligands betacellulin, heparin-binding EGF (HB-EGF), and epiregulin. The last class of EGF-related peptides is composed of neu differentiation factors (NDFs)/heregulines (Holmes et al., 1992), which are ligands for ErbB3 and ErbB4 (Plowman et al., 1993; Carraway et al., 1994). ErbB2 has no known ligand yet, and function by heterodimerization with other EGF receptor family members (Olayioye et al., 2000; Holbro and Hynes, 2004).

When a ligand binds to the EGF receptor it leads to dimerization of two receptors and activation of the intrinsic tyrosin kinase. The dimerization can either be homodimerizaton of two receptors of same type or a hetero dimerization of two different members of the EGF receptor family. An example is that EGF can induce tyrosine phosphorylation of ErB2 through formation of heterodimers of ErbB1 and ErbB2 (King

et al., 1988). Heterodimerization adds an additional level of complexity in signaling from the EGF receptor.

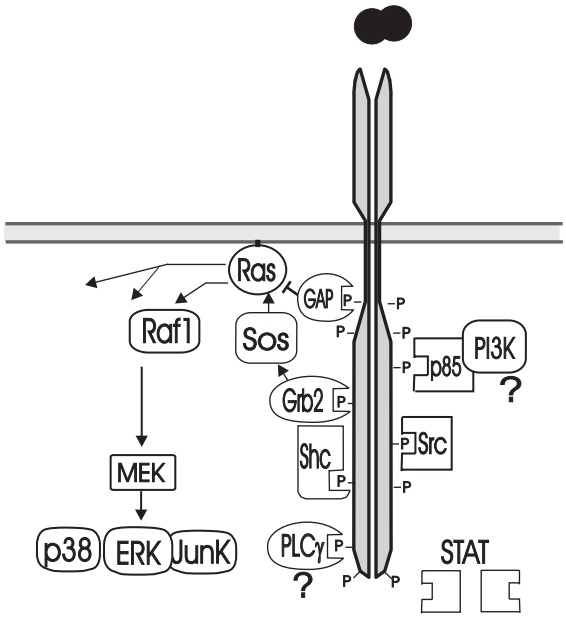
EGF and TGFα both bind and activate the EGF receptor, however certain differences in their biological functions have been shown. In some cells TGFα is more efficient than EGF, and gives a stronger biological respons. This is shown in hepatocytes after stimulation with EGF or TGFα, where TGFα gives a stronger growth respons than EGF (Guren et al., 1996). The mechanisms behind this difference are unknown.

Table 1. EGF receptor and HER family agonists (Harris et al., 2003)	*Receptor*
Epidermal growth factor (EGF)	EGF receptor
Transforming growth factor (TGFα)	EGF receptor
Amphiregulin (AR)	EGF receptor
Epigen	EGF receptor
Heparin-bining EGF-like growth factor	EGF receptor/ErbB4
Epiregulin (EPR)	EGF receptor/ErbB4
Betacellulin (BTC)	EGF receptor/ErbB4
Cripto	EGF receptor/ErbB4
Neuregulin-1 (NRG-1)	ErbB3/ErbB4
Neuregulin-2 (NRG-2)	ErbB3/ErbB4
Neuregulin-3 (NRG-3)	ErbB4
Neuregulin-4 (NRG-4)	ErbB4

Intracellular signaling from the EGF receptor

Ligand binding to the EGF receptors induces dimerization of two receptor, heterodimers or homodimers. Dimerazation leads to proximity of two cytoplasmic tyrosine kinase domains; this allows autophosphorylation of the dimeric partner. Autophosphorylation causes both increased kinase activity by a phosphorylation in the activation loop of the catalytic domain, and phosphorylation of a number non-catalytic sites on the receptor. The tyrosine phosphorylation sites of the non catalytic

Figure 3: Potential signaling pathways from the EGF receptor



residues of the cytoplasmic domain may serve as docking sites for signaling proteins that bind phosphotyrosine or specific protein regions, thus coupling the receptor to various intracellular signaling pathways (Schlessinger, 2000). Signaling proteins involved have specialized domains for recognition of phosphotyrosine: Src homology-2 (SH2) domains and phosphotyrosine binding (PTB) domains binds to tyrosine phosphorylated residues. In addition, SH3 domains that bind proline rich sequences on proteins have been described (Koch et al., 1991; van der Geer and Pawson, 1995). It has been shown that the EGF receptor has several potential substrates that may represent potential pathways, and the role of the different pathways in mitogenic signaling has been extensively studied.

Mitogen Activaded Protein Kinase (MAPK) pathway

MAPKs are important components in intracellular signaling, in particular in mitogenic signaling (Lewis et al., 1998). In hepatocytes the EGF receptor activates MAPKs of the Erk1/Erk2 subtypes, and the extent of the sustained ERK activation in mid/late G_1 phase correlates with the degree of stimulation of DNA synthesis/cellular proliferation (Thoresen et al., 1998). EGF receptor activates Erk1/Erk2 by recruiting Shc and/or Grb2 to the receptor, Shc and Grb2 have no kinase activity themselves. They are adapter proteins which allow the recruitment of the Grb2/Sos complex under the membrane. Sos, the exchange factor of Ras, is then able to activate Ras by facilitate the exchange of GDP for GTP. Activated Ras recruit Raf which activates MEK that phosphorylates Erk1/Erk2 on both threonine and tyrosine residues (Schlessinger, 2000). Activated Erk1/Erk2 is rapidly translocated into the nucleus where it phosphorylates and regulates several transcription factors, including proto-oncogenes c-Fos, and c-Myc (Lewis et al., 1998; Hunter, 2000).

Shc an adapter coupling the EGF receptor to MAPK pathway

Shc is an adapter protein that contains an N-terminal PTB domain, a C-terminal SH2 domain, and a central collagen-like region that contains three tyrosine phosphorylation sites (Koch et al., 1991; Pelicci et al., 1992; van der Geer and Pawson, 1995). In mammals three *shc* genes have been described, their products are referred to as ShcA, ShcB, and ShcC. ShcB and ShcC seem to be limited to neuronal cells in mammals, but

ShcA is ubqiuitously expressed (Pelicci et al., 1996; Ravichandran, 2001). ShcA is expressed as three isoforms, possibly generated through either RNA splicing or alternative translation initiation (Pelicci et al., 1992). The three isforms have different molecular weight of about 46, 52, and 66 kDa, but they are recognized with the same antibody (Ravichandran, 2001). The 52 and 46 kDa isoforms are shown to couple growth factor receptors to the MAPK pathway (Ravichandran, 2001), and is the 52 kDa isoform that is predominantly activated in hepatocytes (data from our lab). The function of the last isoform is more uncertain, but it has been proposed to be a negative regulator the MAPK pathway (Okada et al., 1997).

Shc is activated by tyrosine phosphorylation, and then Grb2 interacts with Shc by a SH2 domain. As described earlier, Grb2 activates MAPK pathway by recruiting Sos to the cell membrane, therefore Shc is coupling the EGF receptor to the MAPK pathway (Schlessinger, 2000).

Signal Transducers and Activators of Transcription (STAT)

The STAT family of signaling proteins is activated by tyrosine phosphorylation, whereby they dimerize and translocate into the nucleus. In the nucleus they bind directly to promoter regions of specific genes and regulate their transcription (Darnell, 1997; Schlessinger, 2000). Nine different STAT proteins have been described in mammals, transcribed from six genes, denoted as Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. The proteins are named as the genes, and in addition Stat1β- and Stat3β-proteins are occurring as alternative splice variants of the respective gene (Bowman et al., 2000). They were first described as mediators for the actions of interferons and other cytokines (Ihle, 2001), but STAT proteins have also been implicated in signaling from growth factor receptors (Leaman et al., 1996).

Intraperitoneal injection of EGF has been shown to activate Stat1, Stat3, Stat5a and Stat5b in mouse liver (Ruff-Jamison et al., 1993), indicating a role in signaling from the EGF receptor. Previous studies in our laboratory showed that in freshly isolated rat hepatocytes, Stat1, Stat3, and particularly Stat5b are activated by EGF. The ability of EGF to induce this activation was attenuated during culturing, while cytokines retained their ability to activate Stat1 and Stat3 (Guren et al., 1999). Src tyrosin kinases have been

implicated in EGF receptor-mediated activation of Stat3 and Stat5 (Olayioye et al., 1999; Wang et al., 1999).

c-Src and signaling from the EGF receptor

The Src family of kinases is intracellular non-receptor tyrosine kinases. A viral form of one of the Src protein tyrosin kinases, namely v-Src from the Rous sarcoma virus, was the first recognized and transforming protein (oncogene). The cellular form of the mutated v-Src is known as c-Src. Both v-Src and c-Src have intrinsic tyrosine kinase acitivity. The Src family of tyrosine kinases comprise of Lck, Blk, Fgr, Lyn, and Hck which are expressed preferentially in hematopoietic cells, while the three c-Src, Fyn, and Yes are more generally expressed. They have been shown to regulate numerous and pleiotropic effects in cells, like cytoskeletal alterations, differentiation, survival, adhesion, and migration (Thomas and Brugge, 1997; Frame, 2002).

The protein structures of the different members of the Src family shows similarities. c-Src is a 60kDa membrane-anchored intracellular protein tyrosine kinase, it is composed of six functional regions: SH4 domain (membrane localizing signal), a unique region (differs between the family members), SH3 domain, SH2 domain, and a catalytic domain. SH3 and SH2 domains are partly responsible for the regulation of the enzymatic activity of c-Src, especially the interaction of the SH2 domain and the phosphorylated tyrosine residue, Y527. When c-Src is phosphorylated on the C-terminal Y527, the SH2-Y527 interaction folds the protein into a less active conformation. Dephosphorylation of Y527 and autophorylation of the Y416 residue in the activation loop of the catalytic domain is essential for full activity of c-Src tyrosine kinase (Thomas and Brugge, 1997).

Many RTKs can recruit and activate Src family kinases, and residues on RTK can themselves be substrates for the Src kinases (Thomas and Brugge, 1997). Thus, Src kinases can theoretically regulate both receptors themselves and downstream signaling proteins. c-Src has been shown to be essential for PDGF induced mitogenesis in some cells (Twamley-Stein et al., 1993). In addition, it has been shown to phosphorylate the EGF receptor on specific tyrosine sites, but c-Src role in mitogenic signaling from the receptor is unclear (Biscardi et al., 1999).

Phosphoinositol-3-kinase

Phosphoinositol 3-kinase (PI 3-K) is activated when binding to p85, an adopter protein containing two SH2 domains and one SH3 domain in addition to the binding site for PI 3-K. p85 couples PI 3-K to receptor tyrosine kinases, and are reported to be phosphorylated in some cell models. But the mechanism leading to activation is not fully understood.

PI 3-K is activated by a number of RTK, but its role in EGF receptor mediated mitogenic signaling has been uncertain (Hu et al., 1992; Raffioni and Bradshaw, 1992). Newer data suggest that it can mediate growth response independently, and it has been shown that inhibitors of PI3K completely blocks EGF induced proliferation (Band et al., 1999; Thoresen et al., 2003). This suggest a mechanism where PI 3-K acts together with ERKs to stimulate growth.

Phospholipase C gamma (PLC-γ)

PLC-γ is a major target for many RTKs (van der Geer et al., 1994), and it can probably bind Tyr-922 and Tyr-1173 on the C-terminal site of the EGF receptor. Its role in EGF receptor signaling is unclear. In hepatocytes, which express a high level of PLC-γ, there is little evidence for EGF receptor induced activation of PLC-γ (Dajani et al., 1999).

G protein-coupled receptors (GPRCs)

GPRCs are receptors coupled to heterotrimeric GTP-binding proteins (G-proteins), and are large family of cell surface receptors characterized by their seven transmebrane domains joined by intracellular and extracellular loops (Ji et al., 1998). GPRCs are coupled to G-proteins which consist of three subunits, Gα,Gβ,and Gγ, which is stably associated when bound to GDP. Ligand binding to the receptors leads to exchange of GDP for GTP in the α-subunit, and the Gβγ dissosates from the GTP-Gα (Hamm and Gilchrist, 1996). Both Gβγ and Gα can mediate cellular effects bye interacting with other proteins. G-proteins are classified by the type of Gα subunit: G_s stimulate adenelyl cyclase and the production of cAMP, and $G_{i/o}$ mediates inhibition of adnelyl cyclase and inactivition of ion channels. G_q is known til couple receptors to activation of PLC-isoforms. The function of Gβγ-subunit is unclear, but regulation of various kinases and cyclases has been shown (van Biesen et al., 1996).

There is evidence that ligands that bind GPRCs can influence cellular proliferation, and GPCR agonists like norephenephrine, vasopressin, angiotensin II, and prostaglandin $F_{2\alpha}$ have in hepatocytes co-mitogenic effects on EGF receptor mediated cellular growth (Dajani et al., 1996; Thoresen et al., 1999). These agonists are mainly thought to act on Gq-coupled GPRCs. A part of this study has explored the underlying signaling mechanism behind the interaction between GPRCs and the EGF-receptor.

Interaction between EGF receptor and GPCRs signaling:

The mitogenic signal from EGF can be modulated by agonists that use GPCRs. GPCR ligands may have co-mitogenic effects on EGF receptor mediated DNA synthesis, but others may have growth inhibitory effects. Co-mitogens are growth factors witch are consider to have no or little mitogenic effect alone, but together with complete mitogens, like EGF and TGFα, they promote cellular proliferation (Michalopoulos, 1990; Thoresen et al., 1990). Angtiotensin II, norephenephrine, vasopressin, and prostaglandin $F_{2\alpha}$ acts as co-mitogens in hepatocytes in primary cultures (Refsnes et al., 1992; Refsnes et al., 1994). There are proposed several theories on the mechanisms behind comitogens; for example crosstalk between intracellular signaling pathways, or regulation on the level of gene transcription.

Cross talk between GPRC and the EGF receptor

The signaling pathways from RTK to Erk1/2 are, as described, well known. It has been shown that various GPCR ligands can induce activation of Erk1/Erk2 (Zwick et al., 1999), but the mechanism behind this cross-talk between the signaling systems is only partly clear. This has received increasing interest in recent years, and several mechanisms have been proposed.

Transactivation of the EGF receptor

It has been found in some cells that GPCR ligands rapidly induce tyrosine phosphorylation of the EGF receptor and activation of downstream signaling pathway leading to Erks (Daub et al., 1997; Zwick et al., 1999). This is an example of cross-talk between the different pathways that provides more diversity in the signaling from the EGF receptor. The mechanism behind GPCR-induced activation of the EGF receptor is

incompletely understood, but the kinetics of EGF transactivation is rapid suggesting involvement of either a tyrosine kinases or increased activity of the EGF receptor kinase itself (Daub et al., 1997; Holbro and Hynes, 2004). The cytoplasmic domain of the EGF recptor has been shown to be phosphorylated by the tyrosine kinases c-Src and Janus kinase 2 (Jak2), thus providing sites for intracellular signaling proteins (Yamauchi et al., 1997; Biscardi et al., 1999). The sites act as scaffolds for the tyrosine kinases, which can activate downstream signaling without the EGF receptor kinase (Holbro and Hynes, 2004). Another theory suggests that EGF receptor transactivation upon GPCR stimulation involve the EGF receptor tyrosine kinase itself. The EGF receptor is stimulated by the autocrine release of EGF receptor ligands, which is mediated by a metalloproteinase activity that is rapidly induced by GPCR ligand interaction. Activity of the metalloproteinase leads to the cleavage of membrane-bound pro-EGF receptor ligands that activate the EGF receptor, resulting in phosphorylation of downstream signaling proteins and activation of Erk (Prenzel et al., 1999). The mechanism of GPCRs induced activation of the metalloproteinase is not known.

Regulation of gene expression

Much of the cellular signaling results in regulation of gene transcription, by activation or deregulation of transcription factors. Transcription factors associated with growth regulates the expression of genes responsible for cell cycle progression (O'Farrell, 1992). Expression of transcription factors and proto-oncogenes c-Myc, c-Fos, and c-Mos have been shown to be increased after stimulation with GPCR ligands in hepatocytes, (Gonzalez-Espinosa and Garcia-Sainz, 1992).

Hepatocytes as a model system

Primary cultures of hepatocytes are useful as a model in the study of regulation of cellular proliferation. Freshly isolated hepatocytes are normal cells with intact receptors and signaling transduction pathways, as opposed to cells that are transformed or transfected. Thus, the hepatocytes may provide experimental information of signal transduction and normal regulation of proliferation which is a useful complement to data obtained in genetically manipulated cells. Also, hepatocytes can be cultured in a serum-free, chemically defined medium (Sand and Christoffersen, 1988), which is important

while serum can contain undefined hormones and growth factors that may influence the regulation of cellular proliferation (Christoffersen et al., 2000).

Hepatocytes as a $G_{0/1}$ to S phase model

Hepatocytes are mainly in early G_1 or in transition from G_0 to G_1 when they are plated. During the next 24 to 48, the cultured hepatocytes progress through the early part of G_1. This may happen independently of growth factors, but the process is dependent on insulin. Insulin is also required for growth responses after exposure to mitogenic growth factors, this effect is mainly evident in early G_1 (Sand et al., 1985). Some ligands that uses GPCRs, for example glucagons, adrenergic agonists, vasopressin, angiotensin II, and prostaglandins, acts as co-mitogens in hepatocytes. They appear to exert their effect by accelerating the traverse through G_1, and thus make more cells responsive to EGF (or other EGF receptor agonists) in mid/late G_1, near the restriction point for further progress and descion to enter S-phase (Pardee, 1989; Michalopoulos, 1990; O'Farrell, 1992). EGF receptor ligands seem to exert their effect in mid/late in G_1 (Sand and Christoffersen, 1987; Loyer et al., 1996; Talarmin et al., 1999).

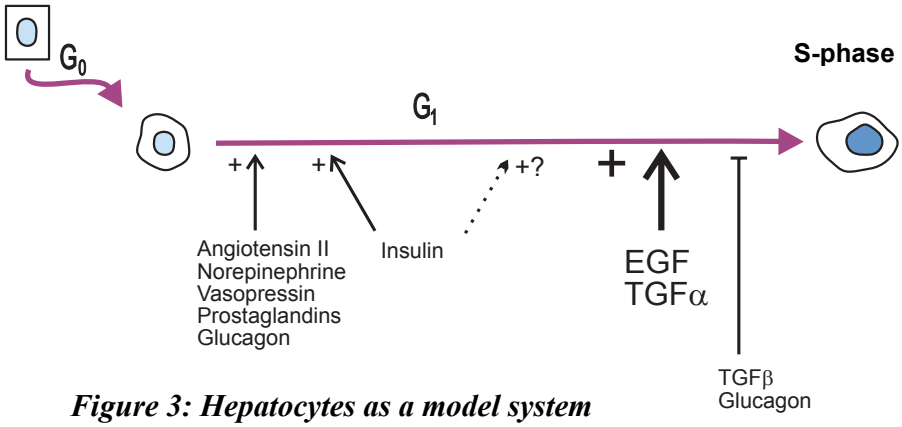


Figure 3: Hepatocytes as a model system

Aims of the study

Stimulation of the EGF receptor mediates growth of hepatocytes by activation of different intracellular signaling pathways, but the mechanisms that regulate these pathways are unclear. This study concerns the activation and regulation of pathways from the EGF receptor, and the interaction between EGF receptors and various GPCR ligands signaling pathways.

More specifically, the study focused on:

1. The mechanisms of EGF receptor mediated activation of STAT proteins, particularly the regulation of Stat5b activation. And Stat5b's role in mitogenic signaling from the EGF receptor.

2. The role of EGF receptor transactivation in the signaling mechanisms involved in growth stimulatory effects of GPRC ligands.

Papers

Two papers are included in this thesis; they are referred to by the numbers they are listed here:

I. Guren T. K., Ødegård J., Abrahamsen H., Thoresen G. H., Susa M., Andersson Y., Ostby E., Christoffersen T. EGF receptor-mediated, c-Src-dependent, activation of Stat5b is downregulated in mitogenically responsive hepatocytes. J Cell Physiol. 2003 Jul;196(1):113-23.

II. Nilsen, L. S., Ødegård J., Thoresen, G.H., Molven, A., Sandnes, D. and Christoffersen T. G protein-coupled receptor agonists stimulate expression of ATF/LRF-1 and c-myc and exert comitogenic effects in hepatocytes without inducing EGF receptor transactivation. J Cell Physiol. 2004. Accepted.

Methods

Isolation and culture of hepatocytes

Male Wistar Rats were fed ad libitum. Hepatocytes were isolated by in vitro collagenase perfusion and low speed sentrifugation (Seglen, 1976) with modifications (Christoffersen et al., 1984). The hepatocytes were seeded onto Costar plastic flask or culture wells depending on the experiment, at cell density of 20 000 per cm^2 to 50 000 cm^2. The culture medium was a serum-free 1:1 combination of Williams's E medium and Dulbecco's modified Eagle's medium with collagen (3 µg/ml), 100 nM insulin, and 25 nM dexamethasone. The medium were supplemented with penicillin (67 µg/ml) and streptomycin (100 µg/ml). The cultures were kept in 95% air/5% CO_2 at 37 ˚C.

Measurement of DNA synthesis

[^{3}H] Thymidine was added to the cultures (1 µCi/ml, 0.125 Ci/mmol) at 24 and 48 hour after plating. Cells were lysed with 0.5 M NaOH at 52 or 72 hours after plating, and DNA was precipitated with trichloricaceticacid before filtration. DNA synthesis was assed by determing the amount of radioactivity cumulatively incorporated into DNA (Refsnes et al., 1994). Protein was determined using Coamassie Plus Protein Assay (Pierce, Rockford, IL, USA).

Immunoprecipitation

The method is based on previously described methods (Yamashita et al., 1998). After agonist stimulation the plated cells were rapidly rinsed twice in ice cold 0.9% NaCl, and once in immunoprecipitation (IP) buffer A (50 mM Tris-HCl, pH 7.4, 280 mM NaCl, 0.2 mM EDTA, 2.0 mM EGTA, 10% glycerol, 1 mM activated Na_3VO_4, 50 mM $Na_4P_2O_7$, 1 mM phenylmetylsulfonyl fluoride (PMSF), and Complete protease inhibitor (Boehringer Mannheim, Germany)). Cells were lysed in 1 ml IP buffer A ,supplied with Nonidet P-40 (NP-40) to final concentration 1%, for 15 min on ice and the lysates were transferred into Eppendorf tubes. Insoluble material was pelleted at 13000 rpm for 10 min at 4 ˚C. Depending on the experiment, clarified lysates (approximately 250 µg protein) were incubated for 60 min with rabbit polyclonal antibodies and the complexes were captured with Dynabeads® (Dynal AS, Oslo, Norway) 0.25 mg per extract, coated with sheep anti-rabbit antibodies (a gift from dr. Kjell Nustad, The Norwegian Radium Hospital, Oslo, Norway). Complexes were washed three times with IP buffer A with 0.5 % NP-40.

Shc proteins were immunoprecipitated under denaturating conditions. After agonist stimulation the cells were lysed inn 500 µl lysis buffer (10 mM Tris-HCl, pH 7.4, 1 % SDS, 1 mM Na_3VO_4), that was boiling when added, then the lysates were boiled for 5 min. After 3 passages through a 27 x G gauge on ice, the lysates were cleared by centrifugation (10 min, 13000 rpm at 4 ˚C). 250 µg of lysate was incubated 2 hours in IP-buffer B (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5 % NP-40 and 1 % Triton-X100) with phosphotyrosine antibody PY-99 (Santa Cruz Biotechnology). The immune complexes were captured with Dynabeads® M-280 sheep anti-Mouse IgG overnight at 4 ˚C, and washed three times with ice-cold IP-buffer B.

The proteins were released from the Dynabeads® by boiling for 5 min in 2x Laemmeli sample buffer. Immunoprecipitates were separated in 8-12%, depending on protein weight, SDS-PAGE gels.

Immunoblotting

Immunopreciptates or aliquots of 20 µg cell protein (prepared in Laemmeli buffer) were separated in 8-12%, depending on protein weight, polyacrylamid (30:1 acrylamide: *N'N'*-bis-acrylamide) gel electrophoresis (PAGE), then transferred to nitrocellulose membranes. Membranes were blocked in T-TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 5% low fat dry milk and 0.05% Tween 20 or T-TBS with 1% bovine serum albumine (BSA) for detection with RC-20 antibody, for 45 minutes, followed by incubation over night at 4 ˚C with primary antibodies. The secondary antibodies, sheep anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase, were incubated in blocking solution for 1.5 hours. The blots were washed in T-TBS for 15 minutes times 3 and then rinsed in water, before they were visualized with Enzyme-linked Chemiluminescence Reagent (ECL, Amersham Pharmacia). Densiometric analysis of immunoblots was obtained with Labworks Software (UVP, Cambridge, UK). Membranes were blocked and reprobed with another antibody after they were washed in H_2O and stripped in 0.5 M NaOH.

Electrophoretic mobility-shift assay (EMSA)

Oligonucleotide probes: Singlestranded oligionucleotides were synthesized at the Biotechnology Center of Oslo, University of Oslo, Norway. Sequence used: 5'-GGA CTT CTT GGA ATT AAG GGA-3', which contains prolactin inducible element (PIE) of the rat β-casein gene and binds Stat5 and Stat1 (Wakao et al., 1994). The oligonucletides were end-labeled using [γ-^{32}P] ATP and 14 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), followed bye annealing to the complementary oligonucleotide sequence to get double-stranded probes. Probes were purified through a 12% non-denaturated PAGE in 0.5 x TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA). The band corresponding to the double stranded probes was detected by autoradiography, cut out of the gel, and eluted in 0.1 x TE-buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) over night. 90% of the water in the eluate was removed by 2-butanol extraction, and the oligonucleotides were stored in 1 x TE buffer in final concentration of 20 fmol/µl. The specific activity of the probes were 5-6000 cpm/fmol.

Preparation of nuclear extracts from cultured hepatocytes: Nuclear extracts were prepared, by a slightly modified version of methods developed for cultured cells (Schreiber et al., 1989; Sadowski and Gilman, 1993). In brief, hepatocytes in primary culture were rapidly washed, and kept on ice for lysis in 1 ml hypotonic buffer A, containing 20 mM HEPES, pH 7.9, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 0.1 mM Na_3VO_4, 50 µM Na_2MnO_4, 1 mM DTT, and Complete Protease Inhibitor (1 tablett per 50 ml solution) for 15 minutes. The cells were scraped with a rubber policeman and collected. Nonidet P-40 was added to the lysates to concentration of 0.20%, then wortexing for 15 sec. The nuclei were pelleted by centrifugation at 10 000g for 30 seconds, and the nuclear pellet was resuspended in 100 µl of a high salt extraction buffer C (buffer A with addition of 420 mM NaCl), under gentle rocking for 30 min.

EMSA: 10 µg of nuclear extracts were preincubated with a binding buffer (13 mM HEPES, pH 7.9, 80 mM NaCl, 8% glycerol, 0.15 mM EDTA, and 1 mM dithioreitol) in presence of 3 µg double stranded poly(dI-dC) (Amersam Biosciences, Buckinghamshire, UK). 1 µl ^{32}P-labeled probe was added to a total volume of 20 µl, and incubated for 30 minutes. Supershift analysis was done by including high-titered polyclonal antibodies against Stat5a, Stat5b or polyclonal Transcruz antibodies against Stat1 (Santa Cruz Biotechnologies) for further 30 minutes, before addition of labeled probes. 10 µl of incubated DNA-protein complexes were loaded on 5% non-denaturated PAGE in 0.5% TBE and electrophorezed at 210 V. The gels were dried and visualized by autoradiography.

Results

Paper I

The first paper is based on the work done in cooperation with Guren T.K. and others, I looked into the mechanisms of EGF recptor induced activation of Stat5, and the significance of this signaling mediator in EGF induced mitogenesis. My main contributions in this paper were the results showing the activation of Stat5b by general and site specific phosphorylation, after stimulation with EGF and other growth factors. I also looked into ligand induced activation of the EGF receptor and the downstream signaling component Shc, and the expression of cycline D1 after stimulation with EGF.

EGF induced activation of Stat5 in hepatocytes

Stat 5a and Stat 5b are activated by phosphorylation of Tyr-694 and Tyr-699 residues, respectively. In cultured hepatocytes we examined the phophorylation of Stat5a and Stat5b after stimulation with EGF and TGFα, and compared it with growth hormone (GH) and prolactin (PRL) induced phosphorylation. Experiments were performed on cultures 3 hours after plating, at a cell density of 20 000 cells per cm^2. Whole cell lysates were immunprecipitated with antibodies against Stat5b and Stat5a, followed by immunoprobing with general anti-phosphotyrosine antibodies (RC-20, PY-20, or 4G10) or phosphospecific antibody recognizing Tyr-699. EGF, TGFα, GH and PRL induced dose dependent phophorylation of Stat5b measured with general antibodies, and it seemed that EGF stimulated a stronger response. But when the immunoblots were probed with Tyr-699 antibodies, GH and PRL gave a more pronounced phosphorylation. Stat5a

was not measurably phosphorylated on Tyr-694 after stimulation with EGF, but slight phosphorylation was detected when probing was performed with general antibodies.

Similar experiments were done with electromobility-shift-assay (EMSA) using the PIE element of the rat β-casein gene as a probe. We analyzed nuclear extracts of the hepatocytes for DNA- binding activity of Stat5. Stimulation of hepatocytes with EGF, PRL, and GH (at 3 hours of culturing) induced dose-dependent increase DNA binding of Stat5b (Stat5a was not detected in these experiments using supershift assays). PRL and GH induced stronger and more persistent activation of Stat5b than EGF. Thus, after EGF stimulation of the hepatocytes cultured for 3 hours the Stat5b phosphorylation and DNA binding activity peaked at 5-15 minutes, before declining and disappering within 60 minutes. The maximum DNA binding activity of Stat5b was less prominent for EGF than PRL and GH, which was in accordance with the phorphorylation of Tyr-699 (see above).

The STAT proteins contain a serine residue between residue 720 and 730 in the C-terminal region which can be phosphorylated, but the function of these residues are uncertain. With an antibody directed towards Ser-730 on Stat5b, we examined immunoblots of imunprecipitates from hepatocytes for serine phosphoryltation. The results indicated that Stat5b was constitutively phosphorlyated on Ser-730, and that this phosphorylation was not affected by treatment with EGF, TGFα, GH or PRL.

c-Src involvement in EGF induced activation of Stat5b

We used a specific inhibitor, CGP 77675, to examine c-Src role in EGF receptor mediated Stat5b activation in hepatocytes (Missbach et al., 2000; Susa and Teti, 2000). CPG77675 was incubated for 90 minutes prior to agonist stimulation of the cells, which resulted in a dose depended inhibition of EGF induced tyrosin phosphorylation and PIE DNA-binding of Stat5b. This inhibition of Stat5b activation was specific for EGF, as CPG77675 did not affect GH or PRL stimulated Stat5b activation. CGP77675 did not inhibit EGF induced phosphorylation of EGF receptor and downstream Shc. Erk1/Erk2 activation was not affected by CGP77675 consentration which completely inhibited Stat5b activation. c-Src appeared to be constitutive active in hepatocytes, detected as phosphorylation of c-Src substrate paxillin. This constitutive activation was completely inhibited with CGP77675 in the same concentration as for Stat5b activation.

Downregulation of EGF induced Stat5b activation in mitogenically responsive hepatocytes

Hepatocytes cultured at high density are known to be less responsive to mitogenic stimuli than low density cultures (Edwards et al., 2000). In our model system hepatocytes cultured at 50 000 cells/cm^2 (high density)shows minute responses on EGF induced DNA synthesis as compared to cultures of 20 000 cm^2 (low density). Also, as described, hepatocytes cultured at optimal density seem to get more responsive for EGF induced mitogensis in mid/late in G_1 phase. This is confirmed by the EGF stimulated DNA synthesis measured over a 40 h period, is greater when EGF is added at 24 h than 3 h after plating. Furtermore, the EGF induced expression of Cyclin D1 are not affected by the timing of addition of EGF, indicating a growth factor dependent restriction point late in G_1 phase.

EGF induced Stat5b activation, measured as PIE DNA binding and phosphorylation of Tyr-699, was in low density cultures more prominent at 3h than 24h after plating, indicating a downregulation. In contrast, in high density cultures the EGF induced activation of Stat5b was more conserved at 24 h after seeding. EGF induced EGF receptor autophosphorylation, Shc and Erk1/Erk2 phosphorylation were preserved at 24 h after plating, as was PRL and GH stimulated Stat5b activation.

GH and PRL did not stimulate DNA synthesis in hepatocytes themselves, and they had no comitgenic effect given as pretreatment (at 3h) on EGF induced DNA synthesis.

Paper II

The study published in the second paper was done in collaboration with Nilssen L.S. and others, and examined the role of EGF receptor transactivation in GPRC agonists modulating of EGF stimulated proliferation. Vasopressin, norepheneprine, angiotensin II, and prostaglandin F2α were used because they act as comitogens on EGF receptor mediated growth in hepatocytes (Dajani et al., 1996; Dajani et al., 1999). In this paper I did the immunoprecipitations of EGF receptor activation and downstream signaling proteins by GPRC agonists.

Pyk2 activation in hepatocytes

Some GPCR can act as comitogens on EGF induced proliferation in hepatocytes, but by them selves have little or no mitogenic effect (Michalopoulos, 1990). The molecular mechanisms behind the comitogenic effect are uncertain, and several theories have been proposed. One explanation can be that GPCRs may activate intracellular signaling cascades which lead to regulation of gene transcription. Pyk2 is non receptor tyrosine kinase, like Src, that is reported to be downstream of GPCRs, and could convey such signal.

Hepatocytes were cultured for 3 h before stimulation with agonists for 5-30 minutes, followed by harvesting of the cells. Immunoprecitations were done on whole cell lysates with antibodies against phosphotyrosine. The immunoprecipitates were separated on PAGE gels and blotted to nitrocellulose membranes before immonoprobing with anti Pyk antibodies.

Stimulation with vasopressin, norepheneprine, angiotensin II, and prostaglandin 2α resulted in phosphorylation of Pyk2, which for vasopressin lasted for at least 30 minutes. EGF induced no detectable activation of Pyk2.

GPCR agonists do not transactivate EGF receptor and proximal downstream signaling mediators

GPCR agonists that act as comitogens stimulate ERK activity with peak activity within 3-5 minutes in hepatocytes (Melien et al., 1998), so transactivation of the EGF receptor should happen inside this timeframe.

Cultures at 3 h after plating were stimulated with the different GPCR agonists for 1-60 minutes, with EGF added as a control. The hepatocytes were harvested and whole cell lysates were immunoprecipitated with EGF receptor antibodies, before separation and immobilizing with western blotting technique. Then the blots were probed with phosphotyrosine antibodies before detection.

The GPCR agonist studied did not induce pshosphorylation of the EGF receptor as an indication of transactiviation, nor did these agonists induce phosphorylation of Shc in similar experiments.

EGF receptor kinase inhibitor AG1478 does not impair GPCR induced Erk1/Erk2 activation

As described, Erk1/Erk2 lie downstream of the EGF receptor, and are important for EGF mediated growth. But Erk1/Erk2 are activated upon stimulation with GPRC agonists, which could possibly implicated involvement of the EGF receptor.

Hepatocytes cultured for 2.5 h before pretreatment with appropriate doses the EGF receptor tyrosine kinase inhibitor AG1478 for 30 min, then at 3 h after plating the hepatocytes were stimulated with the different GPCR agonists or EGF. Western blotting were performed on whole cell lysates, and immunoprobed with phosphor-Erk1/Erk2.

We found that AG1478 dose dependently inhibited EGF stimulated Erk1/Erk2 activity, with complete inhibition at 5μM, but had no effect on GPCR induced activity.

AG1478 does not inhibit the comitogenic effect of GPCR agonists

Comitogens exert their effect on proliferation early in the G_1 phase in hepatocytes. Later in the G_1 phase hepatocytes get mitogenically responsive to EGF (Sand and Christoffersen, 1987; Thoresen et al., 1990). We used AG1478 to examine the role of EGF receptor transactivation on EGF induced DNA synthesis augmented by GPCR agonists.

Primary cultures of hepatocytes were prepared and cultured as described above. GPRC agonists were added to the cultures early in G_1 phase, approximately 3-4 hours after plating, and EGF was added around 24 h after plating. AG1478 was added early or late in the G_1 phase depending on the experiment, ^{3}H-thymidine was added as described. The hepatocytes were harvested at 50-54 h after plating and lysed, followed by precipitation of DNA with acid. Then ^{3}H-thymidine incorporated into DNA was measured after filtration.

Inhibition of EGF receptor with AG1478 late in the G_1 phase completely inhibited EGF induced DNA-synthesis. While addition of AG1478 at the time of stimulation with GPCR agonists had no or little effect on the additive effect of comitogens on measured DNA synthesis.

GPRC agonists induce expression of early response genes independently of EGF receptor signaling

GPRC agonists increase the expression of proto-oncogene c-myc, which appear to be needed for proliferation in hepatocytes (Gonzalez-Espinosa and Garcia-Sainz, 1992; Pelengaris et al., 2002). ATF3 is an other gene that has been shown to have increased expression in regenerating liver (Weir et al., 1994). It may be likely that the comitogenic effect may be dependent on signaling pathways leading directly from GPCR to gene transcription.

Similar experiments as described above were performed on the cultures, but instead of measuring the DNA synthesis the expression of c-myc and ATF3 were done after 1 h ligand stimulation. Gene expression was analyzed bye measuring mRNA transcription with nothern blot analysis.

Vasopressin, norepheneprine, angiotensin II, and prostaglandin 2α induced increased expression of both c-myc and ATF3 in hepatocytes, latter have not been demonstrated before. AG1478 had no effect on the expression of these genes.

Discussion

In this thesis I have included two papers, both where, in collaboration with others in our research group, I have examined different aspects of signaling from the EGF receptor. In in the first of these I looked at the role of EGF receptor activation and some of the proximal downstream signaling mechanisms, and particularly the relationship between STAT proteins and mitogenic signaling.

In the other paper, we studied the regulation of the EGF receptor signaling by GPCR, with particularly focus on the question of whether GPCRs exert growth-promoting effects by transactivating the EGF receptor.

EGF receptor signaling in mitogenic responsive hepatocytes

The EGF receptor can be activated by several ligands as described. Upon ligand binding receptors dimerize and initiate intracellular signaling by binding and activate intracellular

signaling proteins. These proteins are beginnings of the different intracellular signaling pathways witch eventually lead to regulation of biological functions.

In hepatocytes the EGF receptor can activate different signaling pathways, and the role of the different pathways in mitogenic signaling are described earlier. Thus, evidence suggests that two signaling pathways are important in EGF receptor induced proliferation; namely signalling over MAPK pathway and PI3K (Kong et al., 2000; Thoresen et al., 2003). But the roles of other EGF receptor activated pathways in mitogenic signaling have been elusive.

STAT proteins and EGF receptor signaling

Stat1, Stat3, and Stat5 were first reported to be activated by EGF in liver in mice, and studies on hepatocytes and related models suggested that they are involved in regulation of specialized differentiated cellular functions (Runge et al., 1998; Wang et al., 1999). In hepatocytes Stat3 is associated with acute phase responses, and both Stat3 and Stat5 are possibly involved in development and differentiation of hepatocytes. STAT proteins are themselves transcription factors, which bind and regulate genes. Among the targets of Stat1, Stat3, Stat5a, and Stat5b are the genes for c-Myc, cyclin-D1, c-Fos, and $p21^{waf/cip1}$, which are important in the regulation of proliferation (Quelle et al., 1996; Matsumura et al., 1999). This may implicate a role for STAT proteins in growth factor mediated growth signaling.

In hepatocytes expression of Stat1, Stat3, Stat5a, and Stat5b was detected, and Stat1, Stat3, and particularly Stat5b are activated by EGF (Guren et al., 1999). This activation is especially apparent in freshly isolated hepatocytes, which are in the G_0/early G_1 phase of the cell cycle. When the hepatocytes come later in G_1 phase and get mitogenically responsive for EGF, the EGF mediated activation of Stat5b gets less pronounced (Guren et al., 2003) Paper 1.

In paper 1 we examined the mechanism for EGF-induced activation of Stat5b, using primary cultures of rat hepatocytes as a model. EGF-stimulated Stat5b activation was detected as phosphorylation with immunoprecipitation technique and blotting with phosphotyrosine antibodies, and as DNA binding with EMSA. We compared EGF induced Stat5b activation with that of growth hormone (GH) and prolactin (PRL), which

are known to activate Stat5b. EGF-induced a stronger, but more transient, phosphorylation of Stat5b than GH and PRL when we probed the immunopreciptates with a general phosphotyrosine antibody. However, when using an antibody specific for Tyr-699, which is the activation site for Stat5b, we detected a lower degree of phosphorlyation as compared to GH and PRL. This was consistent with the DNA binding of Stat5b, suggesting that EGF receptor phosphorylates residues on Stat5b not involved in activation. The functional role of Stat5b phosphorylation besides Tyr-699 is not known, but it may be involved in negative regulation or another unknown function of Stat5b utilized by the EGF receptor.

Stat5a and Stat5b are known to be phosphorylated on serine residues. This was also apparent in hepatocytes. We found that the serine phosphorylation of Stat5b was constitutive, and was not influenced by addition of EGF, TGFα, PRL, or GH. It is known that Stat5b is serine-phosphorylated on residue Ser-730, and this phosphorylation may be important for the modulation of transcription activity (Yamashita et al., 1998). But in EGF receptor-mediated activation of Stat5b, the role of serine phosphorylation is still unclear.

c-Src may be involved in signaling from RTK as described. In various cell models it has been reported that Src may activate Stat3 and Stat5 (Bromberg et al., 1998; Abram and Courtneidge, 2000; Reddy et al., 2000). Involvement of Src in EGF receptor-mediated activation of Stat5 has also been described (Olayioye et al., 1999). We used the selective Src inhibitor CGP77675 to investigate the role of c-Src in EGF induced activation of Stat5b (Susa and Teti, 2000). The results in paper 1 suggest that c-Src is required for the EGF receptor mediated activation of Stat5b, since addition of the inhibitor blocked the phosphorylation and DNA binding of Stat5b. In contrast, inhibition of c-Src did not affect GH- or PRL-induced Stat5b activation. The basal tyrosine kinase activity of c-Src was not detectably affected by EGF stimulation, but the basal activity was inhibited by CGP77675. This suggests that c-Src is not downstream of the EGF receptor, and that co-operation between the EGF receptor and c-Src is needed for Stat5b activation.

The results in paper 1 also provide more evidence against the involvement of Stat5b in EGF induced mitogenesis in hepatocytes. As described, at the time of seeding

the hepatocytes are mostly in early G_1 phase of the cell cycle, and during culturing they get more sensitive to mitogenic stimulation from EGF. In these experiments the EGF receptor lost its capability to activate Stat5b completely during time in culture, but the receptor retained its ability to activate downstream Shc and MAPK. But PRL and GH preserved their capability to activate Stat5b during culturing. These results, taken together with data showing that hepatocytes cultured at high density maintained their capacity to activate Stat5b after EGF stimulation, suggest that the EGF receptor is uncoupled from Stat5b activation mechanisms when the cells become responsive to EGF-mediated mitogenic stimuli. This may implicate that Stat5b is involved in regulation of functions in the cell that are associated with a higher degree of differentiation, which corresponds with the role of Stat5b in mammary gland development and lactogenesis (Miyoshi et al., 2001). It also produces evidence that the signaling from the EGF receptor is modulated depending upon the state of the cell. In cells that are becoming mitogenically responsive the EGF receptor is uncoupled from a signaling pathway reported to be involved in specialized cellular functions, while retaining and possibly enhancing signaling over pathways responsible for conveying the mitogenic signal.

Regulation of mitogenic EGF receptor signaling by GPCRs

EGF-induced DNA synthesis can be augmented by addition of ligands that bind GPRC in hepatocytes, but the mechanism behind this interaction has been unclear. Recently transactivation of the EGF receptor has been suggested as a possible mechanism for communication between GPRC and EGF receptor. In paper 2 we show that vasopressin, norepheneprine, angiotensin II, and prostaglandin $F_{2\alpha}$, all which enhance the response to EGF, did not induce phosphorylation of the EGF receptor or activation of downstream components. And that GPRC induced activation of Erk1/Erk2 is not influenced by inhibition of the EGF receptor tyrosine kinase. Also, the inhibition of the EGF receptor tyrosine kinase did not prevent the comitogenic effects of GPRC in hepatocytes. Other data show that GPRC-induced ERK activity is not needed for the co-mitogenic effect, as opposed to EGF-induced mitogenesis were ERK activity is essential (Thoresen et al., 2003). This suggests that GPRCs use other mechanisms than EGF receptor transactivation to enhance the EGF-induced proliferation in hepatocytes.

Selective transcription of genes may be a mechanism behind the synergestic effect of GPRC-ligands and EGF on cellular proliferation. Several genes are reported to be activated in hepatocytes following partial hepatectomy and after stimulation with various growth promoting facors (Michalopoulos, 1990). Common for some of the genes are that they may be important for the progression of hepatocytes in the cell cycle. One example is c-myc, which is a proto-oncogene induced by several growth factors. Another example that we have choosed to investigate is ATF3, which has been found to become expressed after partial hepatectomy, and is reported to induce DNA synthesis in mouse hepatoma cells. We examined the role of these two proteins in hepatocytes stimulated with EGF, vasopressin, norepheneprine, angiotensin II, and prostaglandin $F_{2\alpha}$. All GPRC agonists induced expression of c-myc mRNA and ATF3 mRNA, providing a possible mechanism for the growth-promoting effect of these ligands. This effect was independent of EGF receptor transactivation. We also showed that Pyk2 was activated upon stimulation with vasopressin, norepheneprine, angiotensin II, and prostaglandin $F_{2\alpha}$, and may be a part of the downstream signaling pathway from the GPCRs.

Conclusions

1. We found that Stat5b is activated in hepatocytes by EGF detected as tyrosine phosphorylation and DNA binding by a Src dependent mechanism, in contrast to prolactin and growth-hormone induced Stat5b activation. In addition, this activation was downregulated in mitogenically responsive hepatocytes, thus providing evidence that Stat5b is not required for mitogenic signaling from the EGF receptor.

2. The GPCR ligands vasopressin, norepheneprine, angiotensin II, and prostaglandin $F_{2\alpha}$, which acts as co-mitogens on EGF-induced proliferation in hepatocytes, do not exert their effect by transactivation of the EGF receptor. But rather by regulation of gene expression, as these agonists induce transcription of early response genes *c-Myc* and *ATF3*.

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Appendix – Paper I and Paper II

Paper I

JOURNAL OF CELLULAR PHYSIOLOGY 196:113–123 (2003)

EGF Receptor-Mediated, c-Src-Dependent, Activation of Stat5b is Downregulated in Mitogenically Responsive Hepatocytes

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Signal transducer and activator of transcription (STAT) proteins may be activated by epidermal growth factor (EGF), but their role in EGF receptor-mediated mitogenic signaling is not clear. We previously showed that Stat5b was activated by EGF in rat hepatocytes in primary monolayer culture. In the present study, we found that EGF induced tyrosine phosphorylation of Stat5b both on Tyr-699, which correlated with specific DNA binding activity, and also on other tyrosine residues. The Src tyrosine kinase inhibitor CGP77675 blocked the EGF-induced activation of Stat5b, but did not affect the Stat5b activation by growth hormone (GH) or prolactin (PRL). The Stat5b response to EGF was most pronounced soon (3 h) after plating (early G_1) and at high cell density (50,000 hepatocytes per cm^2). However, at this cell density EGF did not stimulate DNA synthesis. In hepatocytes at 24 h of culturing (mid/late G_1) with 20,000 cells per cm^2, EGF induced strong phosphorylation of the EGF receptor, as well as Shc and ERK, and stimulated DNA synthesis, but did not activate Stat5b, although the Stat5b response to GH or PRL was retained. A strong GH-induced Stat5b activation neither influenced the DNA synthesis alone nor enhanced the mitogenic effect of EGF. The results show that EGF induces tyrosine phosphorylation and DNA-binding activity of Stat5b in a manner different from GH and PRL, apparently by a Src-dependent mechanism. The data also provide further evidence that Stat5b is not required for mitogenic signaling from the EGF receptor. J. Cell. Physiol. 196: 113–123, 2003. © 2003 Wiley-Liss, Inc.

The epidermal growth factor (EGF) receptor mediates regulation of cell proliferation and other cellular functions through a complex set of mechanisms that are still not fully understood. Ligand binding elicits receptor dimerization, intrinsic tyrosine kinase activation, and autophosphorylation, resulting in recruitment and activation of various signal molecules (Schlessinger, 2000). A number of proteins that interact with the EGF receptor and may serve as signal mediators have been identified (van der Geer et al., 1994), and some of the downstream signaling pathways have also been delineated, including the Ras-mediated cascade that activates the ERK type of MAP kinases (Chang and Karin, 2001).

Members of the family of 'signal transducer and activator of transcription' (STAT) proteins (Darnell, 1997), first discovered as mediators in the action of interferons and other cytokines (Ihle, 2001), have also been implicated in the mechanisms of signaling from growth factor receptors, including the EGF receptor (Leaman et al., 1996). The STAT proteins are a group of latent, receptor-activated transcription factors, which, upon phosphorylation on a single tyrosine residue, dimerize and translocate to the nucleus, where they

Abbreviations: EGF, epidermal growth factor receptor; STAT, signal transducer and activator of transcription; GH, growth hormone; PRL, prolactin; EMSA, electrophoretic mobility-shift assay; TGFα, transforming growth factor alpha; ERK, extracellular-signal regulated kinase.

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bind to specific sequences in the promoter of target genes (Darnell, 1997). They participate in diverse gene regulation involved in cell differentiation, survival, and proliferation, and possibly also in oncogenesis (Darnell, 1997; Bowman et al., 2000). Seven STAT genes have been identified in mammalian cells, the most widely expressed being Stat1, Stat3, and the highly homologous Stat5a and Stat5b. There is increasing evidence that the different STATs serve distinct functions in the cells (Bromberg and Darnell, 2000). Thus, Stat1 seems to primarily exert antiproliferative effects (Chin et al., 1996; Bromberg et al., 1998a). In contrast, Stat3 may have promitogenic and antiapoptotic effects and has been implicated in malignant transformation in some cells (Bromberg et al., 1999; Song and Rubin Grandis, 2000). Stat5a and Stat5b in hematopoietic cells seem to promote growth, inhibit apoptosis, and may be involved in malignant transformation (Matsumura et al., 1999; Nosaka et al., 1999; Demoulin et al., 2000), and are also essential mediators of the effects of prolactin (PRL) and growth hormone (GH) as well as IL-2 and other cytokines (Groner and Gouilleux, 1995; Grimley et al., 1999; Moriggl et al., 1999; Herrington et al., 2000; Lin and Leonard, 2000). However, although STAT proteins in some cells have been found to be phosphorylated and activated in response to growth factors, including EGF (Ruff-Jamison et al., 1993, 1994, 1995; Richer et al., 1998; Guren et al., 1999; Luetteke et al., 1999; Olayioye et al., 1999), PDGF (Valgeirsdottir et al., 1998; Sachsenmaier et al., 1999), and VEGF (Bartoli et al., 2000), their exact function in receptor tyrosine kinase signaling has not been defined, and the role of STAT proteins in EGF receptor-mediated mitogenic mechanisms is not known.

Hepatocytes in primary monolayer culture is an epithelial model where several mechanisms of hormonal growth regulation, including growth-promoting effects of EGF, have been studied (for review, see Christoffersen et al., 2000). The purpose of the present study was to examine the EGF receptor-mediated activation of Stat5 and its relationship to the mitogenic effect of EGF, using cultured rat hepatocytes as a model. It has been shown that Stat1, Stat3, and Stat5 in mouse liver are activated in vivo upon EGF treatment (Ruff-Jamison et al., 1993), and we found that in cultured rat hepatocytes Stat3 is constitutively active, while only Stat5b is activated by EGF (Guren et al., 1999). In the present study, we have further examined the mechanisms of EGF receptor-mediated activation of Stat5b and its relationship to the mitogenic effect of EGF in cultured hepatocytes. The results show that EGF induces tyrosine phosphorylation and DNA-binding activity of Stat5b in a manner different from GH and PRL, apparently by a Src-dependent mechanism. They also suggest that the activation of Stat5b, in contrast to the EGF-induced stimulation of ERK, is not part of the mitogenic signal from the EGF receptor.

MATERIALS AND METHODS

Materials

EGF (mouse, receptor grade) and ovine PRL were obtained from Sigma (St. Louis, MO). Recombinant human GH was a gift from Pharmacia & Norge AS (Oslo, Norway). The Src inhibitor CGP77675, described in Missbach et al. (1999); Susa et al. (2000) was a gift from Novartis Pharma AG (Basel, Switzerland). Polyclonal rabbit antibodies against Stat5b (C-17, sc-835) and Stat5a (L-20, sc-1081), and EGF receptor (sc-03) were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antisera specific to peptides corresponding to the unique C-termini of Stat5a or Stat5b (Kirken et al., 1997), and antiserum recognizing Stat5a and Stat5b phosphorylated on Ser-725 and Ser-730, respectively (Yamashita et al., 1998), were kindly provided by Dr. Hallgeir Rui, Department of Pathology USUHS, Bethesda, MD. Polyclonal rabbit antibodies against Shc (Cat. No. 06-203) and Stat5a/Stat5b phosphorylated on Tyr-694/Tyr-699 (Cat. No. 05-495) were from Upstate Biotechnology (Lake Placid, NY). Antibodies against phospho-p44/42 MAPK (p-Thr-202/p-Tyr-204) and Cyclin D1 were from Cell Signalling Technology (Beverly, MA). Antibodies against paxillin were from BD Transduction Laboratories (Lexington, KY). The source of other materials has been described (Guren et al., 1996, 1999; Thoresen et al., 1998).

Isolation and culture of hepatocytes and cell lines

Hepatocytes were isolated from male Wistar rats as previously described (Seglen, 1976; Christoffersen et al., 1984). The cells were seeded onto Costar plastic flasks at a density of 20,000 or 50,000 cells per cm^2 and cultured in a serum-free 1:1 combination of Williams' E medium and Dulbecco's modified Eagle's (DMEM) medium with collagen (3 μg/ml), 100 nM insulin, and 25 nM dexamethasone (Thoresen et al., 1998).

Oligonucleotide probes

Single stranded oligonucleotides were synthesized at the Biotechnology Centre of Oslo, University of Oslo, Norway. The following sequence was used: 5′-GGA CTT CTT GGA ATT AAG GGA-3′, containing the PRL-inducible element (PIE) of the rat β-casein gene, which binds Stat5 and Stat1 (Wakao et al., 1994). The oligonucleotides were end-labeled using [γ-^{32}P]ATP (Amersham Biosciences, Buckinghamshire, UK) and a T4 polynucleotide kinase (New England Biolabs, Beverly, MA), followed by annealing to the complementary sequence to obtain double-stranded probes, and purified as described in Guren et al. (1999). The specific activity of the probes was approximately 5–6,000 cpm/fmol.

Preparation of nuclear extracts and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts were prepared as previously described (Guren et al., 1999). In brief, hepatocytes in primary culture were incubated with agonists as indicated in the figure legends, rapidly washed, and kept on ice for lysis in 1 ml hypotonic buffer A, containing 20 mM HEPES, pH 7.9, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 0.1 mM Na_3VO_4, 50 μM Na_2MnO_4, 1 mM DTT, and Complete Protease Inhibitor (1 tablet per 50 ml solution) for 15 min. Monolayers were scraped with a rubber policeman and collected. Nonidet P-40 was added to lysates to a final concentration of 0.20%, followed by wortexing for 15 sec. The nuclei were pelleted by

centrifugation at 10,000*g* for 30 sec, and the nuclear pellet was resuspended in 100 μl of a high salt extraction buffer C (buffer A with addition of 420 mM NaCl), under gentle rocking for 30 min.

EMSAs were performed as described (Guren et al., 1999). In brief, portions (10 μg) of nuclear extracts were preincubated for 15 min in a binding buffer containing 13 mM HEPES, pH 7.9, 80 mM NaCl, 8% glycerol, 0.15 mM EDTA, and 1 mM dithiotreitol, in presence of 3 μg double stranded poly(dI-dC) (Amersham Biosciences, Buckinghamshire, UK). One microlitre of ^{32}P-labelled probe was added to a total volume of 20 μl, and the incubation was continued for 30 min. In supershift analysis of PIE-binding proteins, the experiments were performed by including high-titered polyclonal antibodies against Stat5a or Stat5b (Kirken et al., 1997) or polyclonal Transcruz antibodies against the C terminus of Stat1 (Santa Cruz Biotechnology) for further 30 min, before addition of labeled probes. Ten microlitres of the incubated DNA–protein complexes were loaded on 5% non-denaturated polyacrylamid gels in 0.5× TBE (89 mM Tris-HCl, 89 mM borate, 2 mM EDTA) and electrophorezed at 210 V. The gels were dried and the retarded complexes visualized by autoradiography.

Cell lysis and immunoprecipitaion

The method is based on previously described methods (Yamashita et al., 1998). After agonist stimulation, the plated cells were rapidly rinsed twice in ice cold 0.9% NaCl, and once in immunoprecipitation (IP) buffer A (50 mM Tris-HCl, pH 7.4, 280 mM NaCl, 0.2 mM EDTA, 2.0 mM EGTA, 10% glycerol, 1 mM activated Na_3VO_4, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete protease inhibitor (Roche Diagnostics, Basel, Switzerland, Germany). Cells were lysed in 1 ml IP buffer A supplied with Nonidet P-40 (final concentration 1%) for 15 min on ice and the lysates were transferred into Eppendorf tubes. Insoluble material was pelleted at 13,000*g* for 10 min at 4°C. Depending on the experiment, clarified lysates (approximately 250 μg protein) were incubated for 120 min with 1 μg polyclonal rabbit antibodies and the complexes were captured overnight with Dynabeads (Dynal AS, Oslo, Norway) 0.25 mg per extract, coated with sheep anti-goat antibodies (a gift from Dr. Kjell Nustad, The Norwegian Radium Hospital, Oslo, Norway). Complexes were washed three times with IP buffer A with 0.5% Nonidet P-40.

Shc proteins were immunoprecipitated under denaturating conditions. After agonist stimulation for the time indicated, the cells were lysed in 250 μl boiling lysis buffer (10 mM Tris-HCl, pH 7.4, 2% SDS, 1 mM Na_3VO_4), and the lysates were boiled for 5 min. After 10 passages through a 20× G gauge on ice, the lysates were cleared by centrifugation (19 min, 13,000*g* at 4°C). Two hundred and fifty micrograms of lysate was incubated for 2 h in IP buffer B (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% NP-40, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.2 mM Na_3VO_4) with the phosphotyrosine antibody PY-99 (Santa Cruz Biotechnology). The immune complexes were captured with Dynabeads, coated with sheep anti-mouse antibodies overnight at 4°C, and washed twice with ice-cold IP buffer B. The proteins were released from the Dynabeads by boiling for 5 min in 2× Laemmli sample buffer.

Immunoblots

Immunoprecipitates or aliquots of 20 μg cell protein (total cell lysate prepared in Laemmli buffer) were separated in 8–12%, depending on the protein weight (w/v) polyacrylamide (30:1 acrylamide: *N′N′*-bis-acrylamide) gel electrophoresis (PAGE), and transferred to nitro-cellulose membranes. The filters were blocked in T-TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 5% (w/v) low fat dry-milk and 0.05% Tween-20), followed by incubation overnight at 4°C with primary antibodies. The secondary antibody, horseradish peroxidase-conjugated sheep anti-rabbit IgG, was incubated in blocking solution for 1.5 h. The blots were visualized with Enzyme-linked Chemiluminescence Reagent (ECL, Amersham Pharmacia Biotech). Densitometric analysis of the bands was obtained with the Labworks software package (UVP, Cambridge, UK). For detection of phosphotyrosine, the membranes were blocked in TTBS containing 1% BSA and incubated overnight at 4°C with the anti-phosphotyrosine antibodies RC20 or PY-20 (Transduction Laboratories). When using the 4G10 anti-phosphotyrosine antibody (Upstate Biotechnonlogy, Lake Placid, NY), a protocol recommended by the manufacturer was followed. After detection of tyrosine phosphorylation, the membranes were washed in H_2O, stripped in 0.5 M NaOH, and blocked before reprobing with another antibody.

Other methods

DNA synthesis was measured by thymidine incorporation (Refsnes et al., 1994). [6-^{3}H]-Thymidine (^{3}H-TdR) was added to the cultures (1 μCi/ml, 0.125 Ci/mmol) at 24 and 48 h after plating. Protein contents of nuclear extracts and whole-cell lysates were determined using the Comassie Plus Protein Assay (Pierce, Rockford, IL).

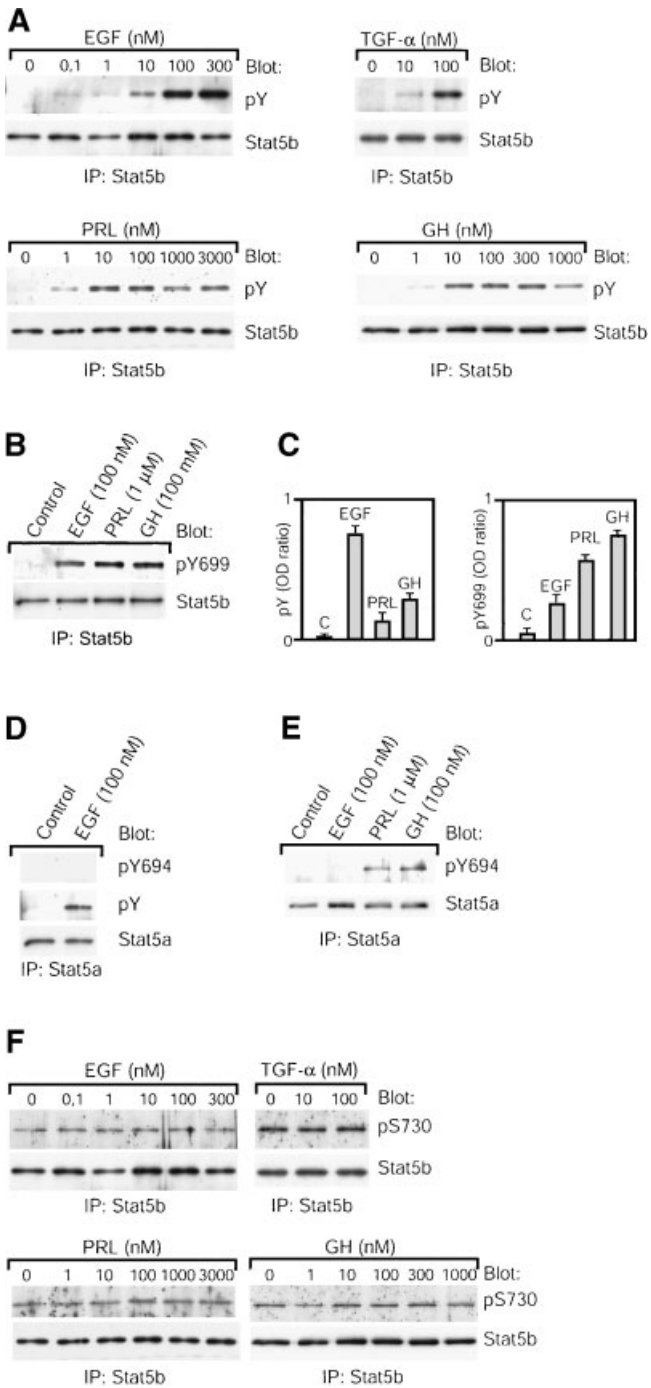
RESULTS

Effect of EGF, TGFα, PRL, and GH on tyrosine phosphorylation of Stat5 in cultured hepatocytes

Phosphorylation of the Tyr-694 and Tyr-699 residues of Stat5a and Stat5b, respectively, leads to activation of the Stat5 proteins (Grimley et al., 1999). We first examined EGF receptor-mediated tyrosine phosphorylation of Stat5a and Stat5b in the hepatocytes, comparing the effects with the responses to PRL and GH. These experiments were performed at 3 h after plating, using a cell density of 20,000 hepatocytes per cm^2.

EGF and TGFα, like PRL and GH, induced tyrosine phosphorylation of Stat5b in the cultured hepatocytes (Fig. 1). After IP from whole-cell lysates, immunoblot probing with general anti-phosphotyrosine antibodies (RC-20, PY-20, or 4G10) showed dose-dependent agonist-induced tyrosine phosphorylation of Stat5b (Fig. 1A and data not shown). The EGF-induced phosphorylation of Stat5b was rapid and transient (see below). Using these general anti-phosphotyrosine antibodies for detection, it was consistently found that EGF produced a larger maximal phosphorylation of Stat5b than did PRL or GH (Fig. 1A,C). However, when the Stat5b immunoprecipitates were blotted with an antibody recognizing

Stat5b phosphorylated on Tyr-699, the EGF-induced phosphorylation generally was of less magnitude than the effects of PRL or GH (Fig. 1B,C). Stat5a, which is also expressed in the primary cultures of rat hepatocytes (Guren et al., 1999), was not measurably phosporylated on Tyr-694 in response to EGF, while a slight phosphorylation was detected by a general phosphotyrosine antibody (Fig. 1D). This may be consistent with the observation that EGF appeared to induce phosphorylation of other tyrosine sites than Tyr-694 in Stat5a (Olayioye et al., 1999). After treatment with PRL or GH a small amount of Stat5a phosphorylated on Tyr-694 was noted (Fig. 1E).



Constitutive serine phosphorylation of Stat5b

The C-terminal region of most STAT proteins contains a serine residue between position 720 and 730, and it has been suggested that phosphorylation of this serine may modulate the transcriptional ability of certain STAT proteins (Decker and Kovarik, 2000). Ser-730 on Stat5b can be phosphorylated (Grimley et al., 1999). The functional importance of this is not clear but there is evidence that phosphorylation of Ser-730 modulates the effects on gene transcription of Stat5b (Park et al., 2001). We examined serine phosphorylation of Stat5b in the hepatocytes, using a specific antibody directed towards Ser-730 (Yamashita et al., 1998). The results indicated that Ser-730 in Stat5b was constitutively phosphorylated in the hepatocytes. However this phosphorylation was not altered significantly upon treatment with EGF, TGFα, PRL, or GH (Fig. 1F).

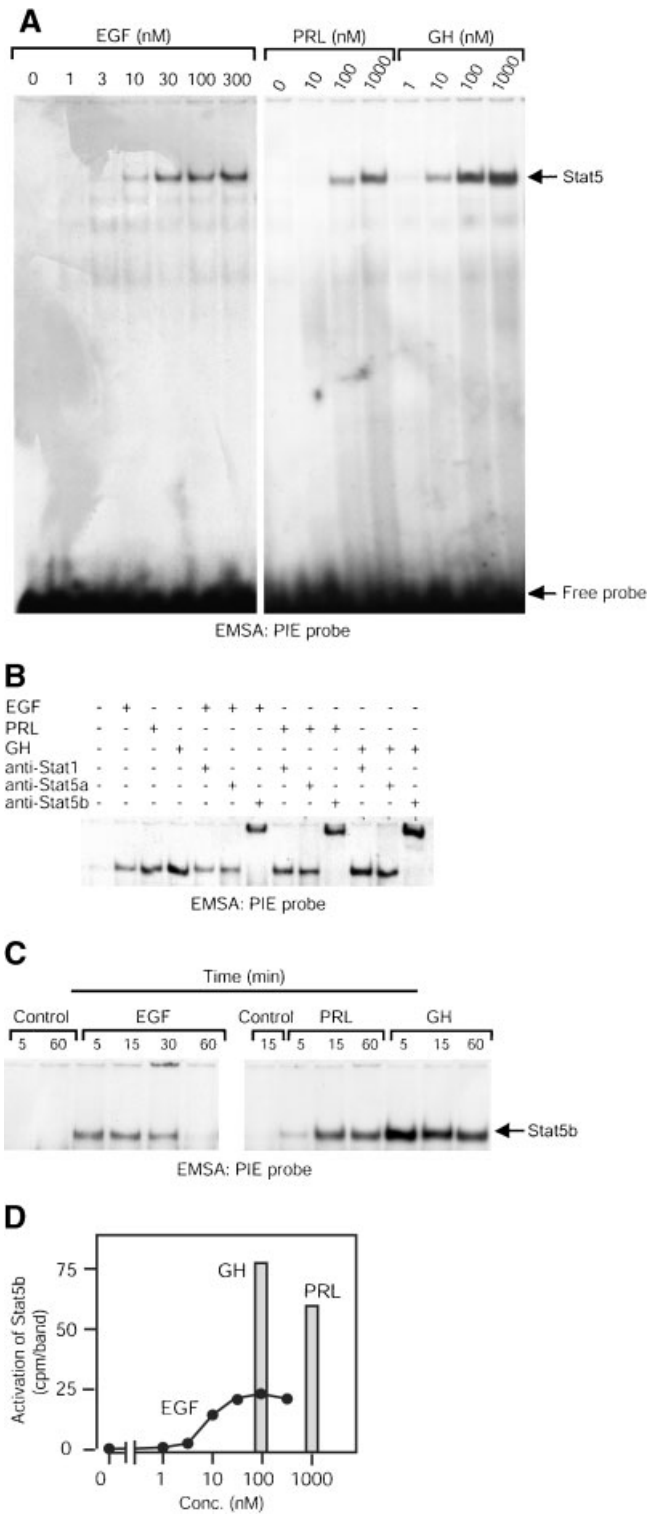
Agonist-induced DNA-binding activity of Stat5b: differential responses to EGF, PRL, and GH

Nuclear extracts of the hepatocytes were analyzed (EMSA) for agonist-induced activation of Stat5, in terms of binding to the PIE of the rat β-casein gene (Wakao et al., 1994). Stimulation of cells (at 3 h of culturing) with EGF, PRL, or GH for 15 min prior to harvesting induced dose-dependent increases in the amount of Stat5 bound to the PIE probe (Fig. 2A). Supershift assays, using antisera that discriminate between Stat5a and Stat5b, raised against their C-terminal parts (Kirken et al., 1997), showed that the EGF-induced PIE-binding complexes consisted of only Stat5b. Thus, these results together with the tyrosine phosphorylation data (Fig. 1) corroborate our previous suggestion (Guren et al., 1999) that EGF selectively activates Stat5b in hepatocytes

Fig. 1. Phosphorylation of Stat5a and Stat5b by EGF, TGFα, PRL, or GH in cultured hepatocytes. Hepatocytes plated and cultured for 3 h at 20,000 cells/cm^2, were stimulated for 15 min with agonist. Stat5a or Stat5b was immunoprecipitated from whole-cell lysates using polyclonal antibodies, separated by SDS–PAGE (10%), and transferred to nitrocellulose membranes. **A**: Tyrosine phosphorylation (pY) was detected by the general anti-phosphotyrosine antibody RC20 directly conjugated to horseradish peroxidase. The membranes were stripped and reprobed with a specific Stat5b antibody (discriminating between Stat5a and Stat5b, Kirken et al., 1997). **B**: The membranes were probed with an antibody recognizing Stat5b phosphorylated on Tyr-699 (pY699), and were stripped and reprobed with a specific Stat5b antibody. **C**: Densitometric analysis of agonist-induced Stat5b tyrosine phosphorylation (pY, left) and Tyr-699 phoshorylation (pY699, right). The hepatocytes were stimulated with EGF (100 nM), PRL (1 μM), or GH (100 nM) and phosphorylation was detected by IP and Western blots as described in A and B. The data (OD ratio; i.e., band density normalized to total Stat5b in arbitrary units) are mean ± SEM of values from three independent experiments. **D**: The membranes were probed with an antibody recognizing Stat5a phosphorylated on Tyr-694 (pY694) and were stripped and first reprobed with a specific a general phosphotyrosine antibody (RC-20) and stripped again before Stat5a was detected with a specific Stat5a antibody. **E**: The membranes were probed with an antibody recognizing Stat5a phosphorylated on Tyr-694 (pY694) and were stripped and reprobed with a specific Stat5a antibody (Kirken et al., 1997). **F**: Upon IP with specific Stat5b antibodies (Kirken et al., 1997), serine phosphorylation was detected with a polyclonal antibody recognizing Stat5b phosphorylated on Ser-730 (pS730) (Yamashita et al., 1998). The membranes were stripped and reprobed with a specific Stat5b antibody.

(Fig. 2B). PRL and GH also activated predominantly Stat5b in the cells. Although we observed a weak phosphorylation of Stat5a on Tyr-694 after PRL or GH stimulation (Fig. 1E) no significant supershift of the Stat5 complexes was detected with a Stat5a antibody (Fig. 2B).

The activation of Stat5b induced by EGF differed from the effects of PRL and GH in being more rapid and transient. Thus, after EGF stimulation both the tyrosine phosphorylation of Stat5b (data not shown) and the PIE-binding activity in nuclear extracts were maximal at 5–15 min and then declined, disappearing within 60 min, while PRL and GH produced more sustained effects (Fig. 2C and data not shown). Quantification of the radioactivity of the retarded Stat5b–PIE complexes in the EMSA gels showed that this effect of EGF was half-maximal at about 10 nM (Fig. 2D). The maximal effect of EGF was significantly lower than the responses to PRL and GH (Fig. 2D), which is in accordance with the difference in phosphorylation of Stat5b at Tyr-699 shown in Figure 1B,C.



c-Src tyrosine kinase inhibitor CGP77675 selectively blocks EGF-induced activation of Stat5b

Src tyrosine kinase seems to be involved in signaling from certain receptor tyrosine kinases and cytokine receptors and may activate Stat3 in some cell models (Bromberg et al., 1998b; Turkson et al., 1998; Biscardi et al., 1999; Abram and Courtneidge, 2000). Src has also been reported to activate Stat5 (Kazansky et al., 1999; Reddy et al., 2000), and recent findings in A431 and HepG2 cells suggest that c-Src may be involved in EGF receptor-mediated activation of Stat5 (Olayioye et al., 1999; Wang et al., 1999), while PDGF-induced Stat5 activation was independent of c-Src and c-Fyn in some transfected cells and cell-free systems (Paukku et al., 2000). To examine the role of c-Src in the effect of EGF on Stat5b activation in the cultured hepatocytes, we preincubated the cells for 90 min with CGP77675, an inhibitor of the c-Src kinase (Missbach et al., 1999; Susa et al., 2000), prior to stimulation with an agonist. CGP77675 dose dependently inhibited EGF-induced tyrosine phosphorylation and PIE-binding activity of Stat5b (Fig. 3A and data not shown), with IC_{50} at about 0.5–1 μM (Fig. 3C), which is in the same range as found for inhibition of tyrosine phosphorylation of Fak and paxillin in Src-overexpressing cells (Missbach et al., 1999).

The inhibition of Stat5 activation by CGP77675 was specific for EGF, as it did not detectably affect tyrosine phosphorylation or the rise in PIE-binding activity induced by PRL or GH (Fig. 3B). Furthermore, the

Fig. 2. Differential effects of EGF, PRL, and GH on DNA-binding activity of Stat5b in cultured hepatocytes. Hepatocytes cultured for 3 h at 20,000 cells/cm^2, were stimulated with EGF, PRL, or GH for 15 min or the time indicated before harvesting. Ten micrograms of nuclear extracts were subjected to EMSA, using a ^{32}P-end labeled oligonucleotide probe from the prolactin-inducible element (PIE) from the rat β-casein gene promoter as a probe (Wakao et al., 1994). **A**: Dose-dependency of Stat5b PIE-binding activity induced by EGF, PRL, and GH. **B**: Supershift analysis. The nuclear extracts were incubated with polyclonal antibodies raised against Stat1, Stat5a, or Stat5b (Kirken et al., 1997) 30 min before the probe was added. The free probe is not shown. **C**: Time courses. Hepatocytes were stimulated with EGF (100 nM), PRL (1 μM), or GH (100 nM). Nuclear extracts were subjected to EMSA. The free probe is not shown. **D**: Retarded bands from EMSA were cut out of the dried gels. The radioactivity of the bands was quantified in a scintillation counter (graph).

CGP77675 did not inhibit EGF-induced tyrosine autophosphorylation of the EGF receptor (Fig. 3D). Neither did preincubation with CGP77675 at 1–2.5 μM, i.e., concentrations that completely inhibited EGF-induced tyrosine phosphorylation and PIE-binding activity of Stat5b, affect the EGF-induced phosphorylation of ERK, which required ≥10 μM of CGP77675 for significant inhibition (Fig. 3E).

EGF-induced Stat5b activation is downregulated in mitogenically responsive cultured hepatocytes

Previous studies have shown that hepatocytes cultured at high cell densities are less responsive to mitogenic stimulation than low-density cultures (Nakamura et al., 1983; Edwards and Michalopoulos, 2000). This is also shown in Figure 4A, which compares the DNA synthesis in hepatocytes at 20,000 and 50,000 cells per cm^2. Furthermore, the hepatocytes (when cultured at an optimal density) become more responsive and sensitive to mitogenic stimulation by EGF when they have traversed the first part of the G_1-phase (Sand and Christoffersen, 1987; Wollenberg et al., 1989; Loyer et al., 1996), apparently reflecting a requirement for the mitogen at a restriction point in mid/late G_1 (Loyer et al., 1996). Consistent with this, Figure 4B,C shows that in different EGF-treated cultures cyclin D1 is expressed at 42 h after plating, irrespective of whether EGF is added at 3 or 24 h, and that the DNA synthesis (measured as ^{3}H-TdR incorporation during a 40 h period) is markedly larger with addition of EGF at 24 h as compared to 3 h. Thus, low-density cultures (≤20,000 cells per cm^2) at a late point (≥24 h) of culturing represent hepatocytes that are responsive to the mitogenic effect of EGF in contrast to high density and/or early cultures.

Figure 4 shows that when the hepatocytes were cultured at a density of 50,000 cells per cm^2, EGF did not significantly stimulate DNA synthesis; however, in these cultures Stat5b was clearly phosphorylated on Tyr-699 and activated by EGF, and, in particular, the ability of the cells to produce a Stat5b response to EGF was retained for at least 24 h in these high-density cultures. In contrast, in cultures with 20,000 cells per cm^2, exposure to EGF at 24 h after seeding efficiently stimulated the DNA synthesis (Fig. 4A,C) but did not elicit any measurable effect on Stat5b, in terms of phosphorylation at Tyr-699 or DNA-binding activity (Figs. 4D,E and 5A,B), although tyrosine phosphorylation of Stat5b was detected with a general phosphotyrosine antibody, suggesting phosphorylation on other tyrosine residues than Tyr-699 upon EGF stimulation (Fig. 5B). Thus, when the hepatocytes were in a state where they were highly responsive to the mitogenic stimulus, i.e., in low-density cultures at 24 h after plating, the effect of EGF on Stat5b was downregulated. This downregulation of mechanisms activating Stat5b was selective for EGF, since PRL and GH markedly phosphorylated and activated Stat5b under the same conditions (Fig. 5A,C and data not shown). Furthermore, EGF did not influence the GH-induced activation of Stat5b (Fig. 5C).

At 24 h in low-density cultures, EGF induced a rapid autophosphorylation of the EGF receptor (Fig. 5D), and, in contrast to the lack of Stat5b activation, several other EGF receptor-mediated signaling mechanisms were sustained in the mitogenically responsive hepatocytes.

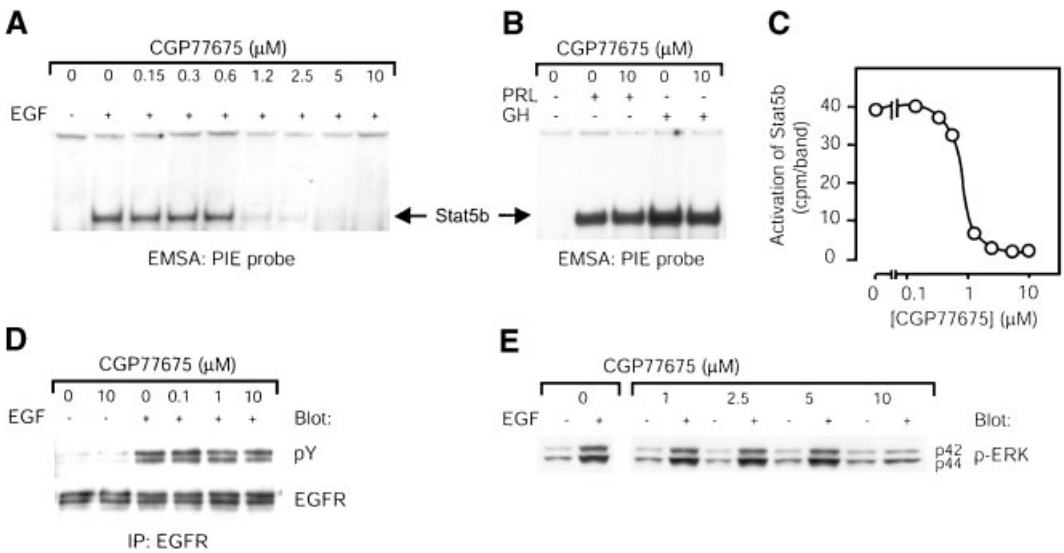


Fig. 3. Selective inhibition of EGF-induced activation of Stat5b by the Src tyrosine kinase inhibitor CGP77675. Hepatocytes cultured for 3 h at 20,000 cells/cm^2 were stimulated with agonists after 90 min pretreatment of the Src tyrosine kinase inhibitor CGP77675 or DMSO (solvent, in control cells). **A**, **B**: Nuclear extracts obtained after stimulation with EGF (10 nM), PRL (1 μM), or GH (100 nM) for 15 min were subjected to EMSA, using a ^{32}P-end labeled PIE as probe. The free probe is not shown. Pretreatment with DMSO had no effect on the EGF-induced Stat5b responses; neither did CGP77675 alone affect Stat5b (data not shown). **C**: The retarded complexes bands of A were cut out of the dried gel and the radioactivity was counted (graph). **D**: EGF receptor was immunoprecipitated from whole-cell lysates from hepatocytes stimulated with EGF (10 nM) by polyclonal antibodies against the EGF receptor, separated in SDS–PAGE (8%), and transferred to nitrocellulose membranes. Tyrosine phosphorylation (pY) was detected by the phosphotyrosine antibody 4G10. The membranes were stripped and reprobed with an EGF receptor antibody. **E**: Whole-cell lysates from hepatocytes stimulated with EGF (10 nM) for 5 min were separated by SDS–PAGE (12%), transferred to nitrocellulose membranes. ERK phosphorylation was detected by a specific antibody against double phosphorylated (active) ERK.

Thus, we found a marked EGF-induced phosphorylation of p52/Shc and p46/Shc (Fig. 5E). Furthermore, treatment with EGF caused a rapid phosphorylation of ERK in the mitogenically responsive hepatocytes (Fig. 5F), and, in agreement with our previous observation (Guren et al., 1999), elicited a strong increase in ERK activity. It should be noted that the magnitude of the Shc or ERK responses did not decrease from 3 to 24 h (Fig. 5). Other experiments have shown that inhibition of a sustained EGF-induced ERK activation blocks the DNA synthesis (Thoresen et al., 2003).

GH (Fig. 6) and PRL (data not shown) did not stimulate DNA synthesis, and pre-treatment with GH (at 3 h) did not enhance the mitogenic effect of EGF (added at 24 h, Fig. 6). This provides further evidence that activation of Sta5b is not required for stimulation of DNA synthesis in hepatocytes.

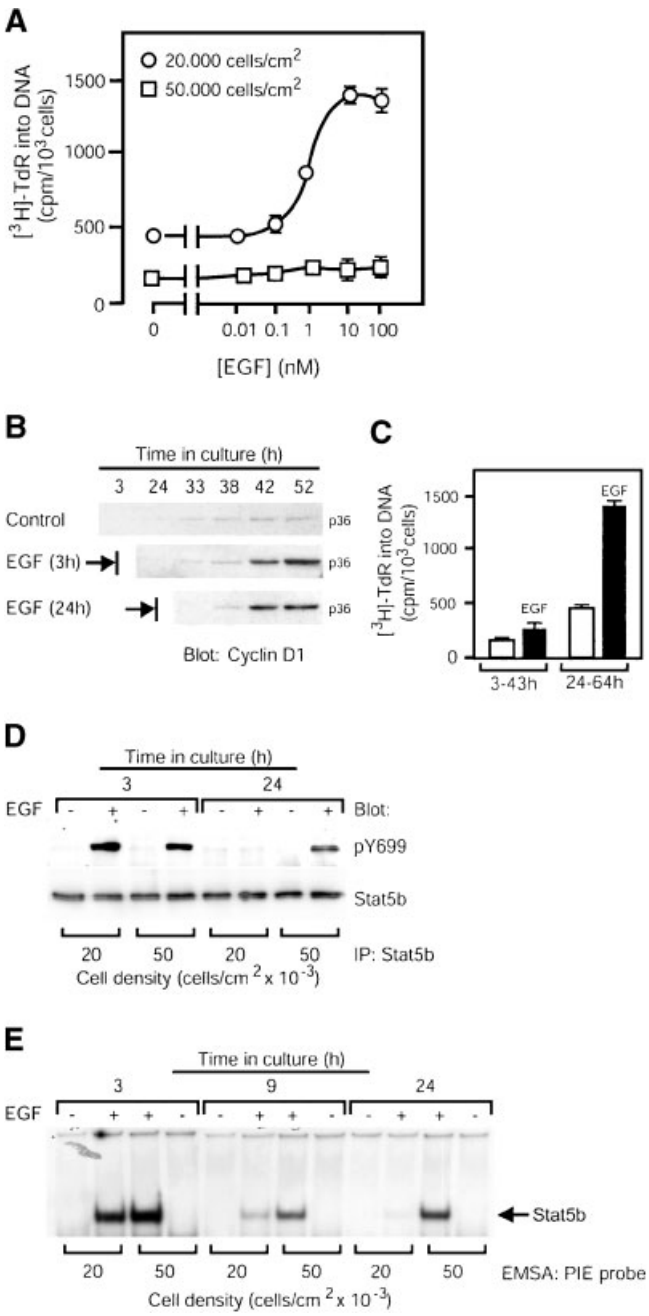


Fig. 4. EGF-induced activation of Stat5b and DNA synthesis at different culturing conditions. Hepatocytes cultured at low (20,000 cells/cm^2) or high (50,000 cells/cm^2) density for various time periods. **A**: Hepatocytes precultured for 24 h were stimulated with EGF. ^{3}H-thymidine incorporated into DNA was assessed in the following 48 h. Data are the mean ± SEM of triplicates cultures in a typical experiment. **B**: Expression of Cyclin D1 induced by (10 nM) addition of EGF at 3 or 24 h after plating. The cells were harvested at the time indicated, whole-cell lysates were analyzed by immunoblotting with an antibody against Cyclin D1. **C**: DNA synthesis induced by EGF (10 nM) addition at 3 or 24 h after plating, assessed as ^{3}H-thymidin incorporation in the period 3–43 h or 24–64 h, respectively (mean ± SEM of triplicate cultures from a representative experiment). **D**: Hepatocytes cultured for 3 or 24 h were stimulated with EGF (100 nM) for 15 min and harvested. Lysates were immunoprecipitated with Stat5b antibodies (Kirken et al., 1997) and tyrosine phosphorylation was detected in Western blots by an antibody recognizing Stat5b phosphorylated on Tyr-699 (pY699). The membranes were stripped and reprobed with a specific Stat5b antibody. **E**: Hepatocytes cultured for 3, 9, or 24 h were stimulated with EGF (100 nM) for 15 min and harvested. Nuclear extracts were subjected to EMSA, using a ^{32}P-end labeled PIE as probe. The free probe is not shown.

DISCUSSION

The role of STAT proteins in the signaling from growth factor receptors is not clarified. Although EGF receptor-mediated activation of Stat5 has been observed, its function in epithelial cells is not well understood. The present study, using rat hepatocytes in primary monolayers as the cell model, provides further evidence that EGF can stimulate tyrosine phosphorylation and induce specific DNA-binding activity of Stat5b. Our results show that the pattern of phosphorylation and activation of Stat5b induced by EGF in hepatocytes differs qualitatively from the effects of GH and PRL and apparently was Src-dependent. Furthermore, the study also provides evidence against a requirement for EGF-induced Stat5b activation in mitogenic signaling from the EGF receptor in these cells.

When immunoprecipitates were probed with a general phosphotyrosine antibody, EGF was found to cause a tyrosine phosphorylation of Stat5b that was more pronounced than the effects of PRL and GH, but was transient. In contrast, specific Tyr-699 phosphorylation and DNA-binding activity of Stat5b elicited by maximally effective concentrations of EGF were of lower magnitude than the responses to PRL or GH. Also, under conditions where the EGF-induced activation of Stat5b was downregulated, tyrosine phosphorylation of Stat5b could nevertheless be detected with a general phosphotyrosine antibody. Taken together, these results suggest that EGF, but apparently not PRL or GH, may induce phosphorylation of tyrosine residues other than Tyr-699 in Stat5b. Recently the Tyr-725, -740, and -743 of Stat5b were identified as residues that are phosphorylated in response to EGF in transfected GHR293 cells (Kloth et al., 2002). The functional role of the phosphorylation of the additional Stat5 tyrosine residue(s) is not known, but could be involved in negative regulation of transcription (Kloth et al., 2002) or reflect a function of Stat5 utilized by the EGF receptor but not related to DNA binding activity.

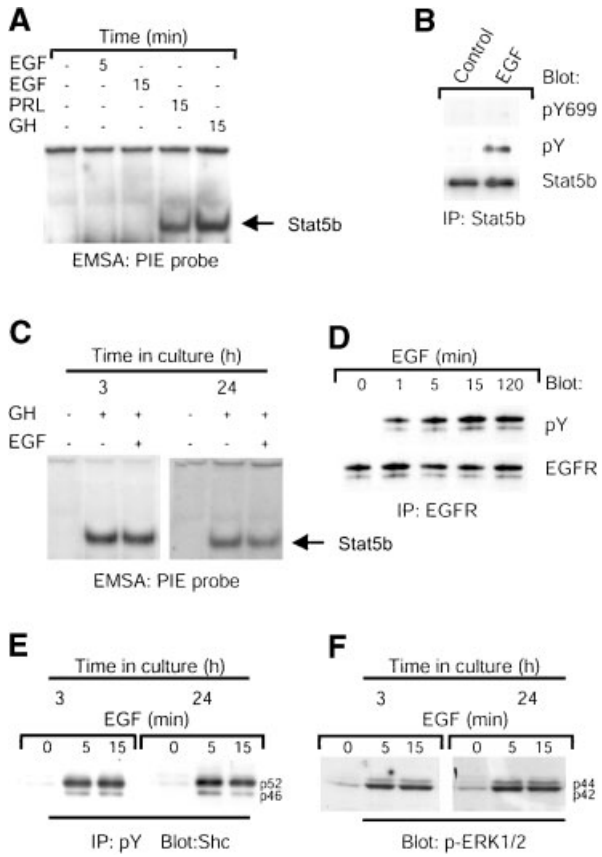


Fig. 5. Selective, time-dependent downregulation of EGF-inducible Stat5b activation in cultured hepatocytes. Hepatocytes were cultured at a density of 20,000 cells/cm^2. **A**: Nuclear extracts from cells cultured for 24 h and stimulated with EGF (100 nM), PRL (1 μM), or GH (100 nM) for the time indicated were subjected to EMSA, using a ^{32}P-end labeled PIE as probe. The free probe is not shown. **B**: Hepatocytes cultured for 24 h were stimulated with EGF (100 nM) for 15 min and harvested. The lysates were immunoprecipitated with Stat5b antibodies (Kirken et al., 1997) and tyrosine phosphorylation was first detected by an antibody recognizing Stat5b phosphorylated on Tyr-699 (pY699). The membranes were stripped and first reprobed with a general phosphotyrosine antibody (RC-20), stripped again before being reprobed with a specific Stat5b antibody. **C**: Nuclear extracts from cells (cultured for 3 or 24 h) that had been stimulated with GH (100 nM) alone or in combination with EGF (10 nM) for 15 min were subjected to EMSA, using a ^{32}P-end labeled PIE as probe. The free probe is not shown. **D**: Hepatocytes were cultured for 24 h and stimulated with EGF (10 nM) for the time indicated. EGF receptor was immunoprecipitated from whole-cell lysates using polyclonal EGF receptor antibodies. Tyrosine phosphorylation (pY) was detected in Western blots by the 4G10 phosphotyrosine antibody. The membranes were stripped and reprobed with an EGF receptor antibody. **E**: Hepatocytes were cultured for 3 or 24 h and stimulated with EGF (10 nM) for the time indicated. Shc proteins were immunoprecipitated with the phosphotyrosine antibody PY-99 under denaturating conditions from whole-cell lysates. Shc proteins were detected in Western blots using antibodies recognizing the three forms of Shc (p46/Shc, p52/Shc, and p66/Shc). **F**: Hepatocytes were cultured for 3 or 24 h and stimulated with EGF (10 nM) for the time indicated. Whole-cell lysates were separated on 12% SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies against double phosphorylated (active) ERK.

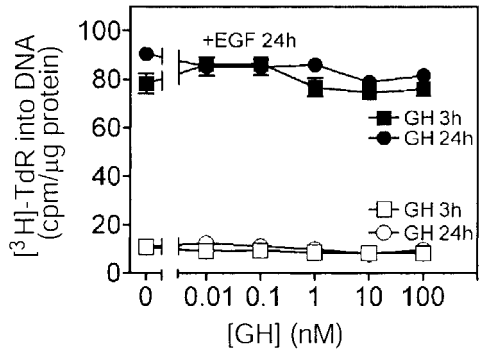


Fig. 6. Lack of effect of GH on hepatocyte DNA synthesis. Hepatocytes were cultured at 20,000 cells/cm^2. GH was added at 3 or 24 h after hepatocyte plating. ^{3}H-thymidine incorporated into DNA was assessed from 24 to 65 h after plating in absence (open symbols) or presence of 10 nM EGF (added at 24 h, filled symbols). Data are mean ± SEM of triplicates cultures in a typical experiment.

There are data suggesting that STAT proteins may have a role in cellular actions of Src (Bromberg et al., 1998b; Turkson et al., 1998; Garcia et al., 2001). The present results with the inhibitor CGP77675, suggested that c-Scr tyrosine kinase is selectively involved in the EGF-induced activation of Stat5b in hepatocytes. These findings obtained in normal cells are in agreement with results indicating a role of c-Src tyrosine kinase in the EGF receptor-mediated activation of Stat5 in A431 and HepG2 cells (Olayioye et al., 1999; Wang et al., 1999). We have observed that EGF induces tyrosine phosphorylation of paxillin, which may be a downstream response to c-Src (Missbach et al., 1999), and was sensitive to pharmacological inhibition of c-Src, and this effect was more pronounced in high-density cultures (data not shown). We also observed a basal tyrosine kinase activity in immunoprecipitates of c-Src from hepatocyte lysates, which was inhibited by CGP77675, but so far we have not found any significant increase of this kinase activity after EGF stimulation (data not shown). The data support the notion that although c-Src tyrosine kinase activity is required for EGF-induced Stat5b activation, this might reflect a cooperation between c-Src and the EGF receptor and not necessarily c-Src activation as a direct downstream effect of the EGF receptor (Biscardi et al., 1999; Abram and Courtneidge, 2000; Reddy et al., 2000; Garcia et al., 2001).

The present results do not support a role of Stat5b in the mitogenic action of EGF in hepatocytes. Stat5 proteins have been implicated in stimulation of cell growth, inhibition of apoptosis, and oncogenic development in certain other cells (Bowman et al., 2000). Among the targets of Stat5a and Stat5b are the genes of cyclin D1, *p21$^{waf1/cip1}$*, *c-Fos*, and *c-Myc* (Quelle et al., 1996; Matsumura et al., 1997, 1999). At the time of seeding, most of the hepatocytes are in the early G_1 phase of the cell cycle (Christoffersen et al., 2000). During culturing, the hepatocytes progress to a restriction point in mid/late-G_1 where they become highly responsive to mitogenic stimulation by EGF (Sand and Christoffersen, 1987; Wollenberg et al., 1989; Guren et al., 1996; Loyer et al., 1996; Talarmin et al., 1999; Christoffersen et al., 2000). In these cells, the EGF-induced activation of Stat5b was completely downregulated, but EGF readily elicited tyrosine phosphorylation of the EGF receptor and of the adapter protein Shc and induced activation of ERK. We have also observed that under these conditions

EGF stimulation results in activation of the p38 and JNK subtypes of MAP kinases, as well as PI 3-kinase, and pharmacologic blockade of ERK, p38, or PI 3-kinase inhibits EGF-induced DNA synthesis (Thoresen et al., 2003). Supplementation of the EGF stimulation with a strong (GH- or PRL-induced) Stat5b activation did not enhance the DNA synthesis. Furthermore, when hepatocytes were plated at a high density, they became unresponsive to mitogenic stimulation with EGF, but in these cultures the mechanism of EGF receptor-mediated activation of Stat5b was sustained. Together these results suggest that Stat5b is not a part of the mitogenic signal from the activated EGF receptor in the hepatocytes.

It was recently reported that Stat5b, like Stat3, may potentiate v-Src-mediated transformation by stimulating quiescent (G_0) NIH 3T3 cells to enter the G_1 phase (Kazansky and Rosen, 2001). In our experiments, GH-induced activation of Stat5b in hepatocytes at 3 h of culturing did not influence the DNA synthesis by itself, neither did it enhance the mitogenic effect of EGF in mid/late G_1 cells. These results do not support a promitogenic role of Stat5b activation in early G_1 in the cultured hepatocytes, in contrast to other mechanisms such as protein kinase C-mediated signals that accelerate traverse through early G_1 and increase the response to EGF (Dajani et al., 1999; Christoffersen et al., 2000).

In some cells, Stat5 proteins appear to have an anti-apoptotic effect (Sillaber et al., 2000), apparently by regulation of *Bcl-XL* gene expression (Socolovsky et al., 1999; Gesbert and Griffin, 2000). An increased amount of Stat5 activity has been reported in breast cancer cells (Watson and Miller, 1995) and it has been suggested that Stat5a is a survival factor for mammary glands that may participate in tumorigenesis (Humphreys and Hennighausen, 1999). In mouse hepatocytes, EGF induces transcription and protein expression of *Bcl-XL*, but the activating mechanism is not clear (Musallam et al., 2001). Whether or not Stat5b proteins are involved in this mechanism requires further examination. Preliminary experiments from our laboratory have so far not shown an EGF-induced expression of *Bcl-XL* or *Bcl-2* in cultured rat hepatocytes.

The specific Tyr-699 phosphorylation and activation of Stat5b elicited by EGF was of lower magnitude than the effects of GH and PRL. There was also a lower sensitivity for EGF-induced activation of STAT proteins ($ED_{50} = 10$ nM) compared to other effects of EGF, such as stimulation of ERK and of DNA synthesis ($ED_{50} = 1$ nM, see e.g., Thoresen et al., 1998). Thus, a strong tyrosine phosphorylation of Stat5b seems to require a high concentration of EGF or, as recently published, overexpression of EGF receptors (Kloth et al., 2002). It is conceivable that differential sensitivity of EGF receptor responses may represent a determinant of specificity in the EGF receptor signal transduction, depending on the cellular context. The majority of EGF receptor ligands generally act over short distances as autocrine or paracrine growth factors (Olayioye et al., 2000).

Reports from hepatocytes and hepatocyte-related models have suggested that STAT proteins, including Stat5, are involved in functions associated with a high degree of cell differentiation (Runge et al., 1998; Wang et al., 1999), in analogy with the role of Stat5 in mammary gland development and lactogenesis (Miyoshi et al., 2001).

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Paper II

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G Protein-Coupled Receptor Agonist-Stimulated Expression of ATF3/LRF-1 and c-myc and Comitogenic Effects in Hepatocytes do not Require EGF Receptor Transactivation

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Several agonists acting on G protein-coupled receptors (GPCR) enhance the mitogenic effect of epidermal growth factor (EGF) in rat hepatocytes, through mechanisms that have only partially been clarified. Results in various cells have led to the idea that a major mechanism for GPCR-mediated stimulation of cell growth is transactivation of receptor tyrosine kinases, particularly the EGF receptor (EGFR), leading to rapid phosphorylation of the EGFR and activation of downstream signaling pathways. In the present study cultured rat hepatocytes were exposed to various GPCR agonists, including vasopressin, angiotensin II (Ang.II), norepinephrine, or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). None of these agents increased the phosphorylation of the EGFR or the docking protein Shc. Furthermore, we examined the effect of the GPCR agonists on the expression of two early response genes believed to be involved in growth activation. The GPCR agonists increased the mRNA expression of c-myc, and also of activating transcription factor 3 (ATF3)/liver regeneration factor-1 (LRF-1), which is a novel finding. Finally, the selective EGFR inhibitor AG1478 did not suppress the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) or the induction of c-myc or ATF3/LRF-1 by the GPCR agonists, and did not prevent the comitogenic effects induced by these agents, while it blocked the effect of EGF on these responses. The results suggest that GPCR agonists induce expression of ATF3/LRF-1 and c-myc and exert comitogenic effects through mechanisms that do not require EGFR transactivation. J. Cell. Physiol. 201: 349–358, 2004.

G protein-coupled receptors (GPCRs) can mediate enhancement of growth in various cell types (van Biesen et al., 1996; Dhanasekaran et al., 1998). In hepatocytes, GPCR agonists are only weakly mitogenic alone, but a number of these agents are able to enhance the effect of mitogens such as epidermal growth factor (EGF) and hence have been termed comitogens (Michalopoulos, 1990). While the mitogenic effect of EGF and other receptor tyrosine kinase (RTK)-activating growth factors is exerted in the mid/late G_1 phase in cultured rat hepatocytes (Sand and Christoffersen, 1987; Christoffersen et al., 2000) and is required for further progression and S phase entry (Loyer et al., 1996; Talarmin et al., 1999), comitogenic stimulation by GPCR agonists in these cells is exerted primarily in early G_1 and results in acceleration of the traverse through the first part of G_1 (Thoresen et al., 1990; Refsnes et al., 1992; Dajani et al., 1996; Christoffersen et al., 2000). There is evidence suggesting that multiple mechanisms, involving different G proteins and downstream pathways, may participate in conveying the comitogenic signals (Christoffersen et al., 2000). Unlike mitogenic signaling via the EGF receptor (EGFR), comitogenic GPCR-mediated stimulation appears to be independent of activation of extracellular signal-regulated kinase (ERK) in the hepatocytes (Nilssen et al., 2002). Some GPCR agonists have also been found to induce the

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expression in the hepatocytes of certain early response genes that have a role in transcription programs necessary for cell growth, such as *c-fos*, *c-mos*, and *c-myc* (Gonzalez-Espinosa and Garcia-Sainz, 1992). However, the exact mechanisms of the growth-promoting effects of GPCR agonists and how they differ from RTK-mediated mitogenic signaling are not clarified.

One idea that currently is widely advocated is that a major mechanism for GPCR-mediated stimulation of cell growth is transactivation of RTKs (particularly of the EGFR), in terms of a detectable increase in RTK phosphorylation, and activation of downstream signaling molecules shortly after stimulation with GPCR agonists (Eguchi and Inagami, 2000; Prenzel et al., 2000; Gschwind et al., 2001). Support for this hypothesis comes from observations in cell models where several GPCR agonists have been found to elicit rapid EGFR phosphorylation (Daub et al., 1996; Eguchi et al., 1998; Pai et al., 2002) and activation of downstream signaling pathways, including association of the adaptor proteins Shc and Grb2 with the EGFR (Luttrell et al., 1997; Eguchi et al., 1998; Shah and Catt, 2002). Furthermore, by the use of selective EGFR kinase inhibitors, or overexpression of a dominant negative EGFR mutant, ERK activity and DNA synthesis in response to GPCR ligands can be suppressed (Daub et al., 1996; Eguchi et al., 1998; Santiskulvong et al., 2001; Chiu et al., 2002; Kodama et al., 2002; Pai et al., 2002). Although the underlying mechanisms are not fully defined, intracellular signals involving Ca^{2+}, protein kinase C (PKC), and the nonreceptor intracellular tyrosine kinases Src and Pyk2, have been implicated as early intermediates in the EGFR transactivation pathway (Eguchi et al., 1998, 1999; Luttrell et al., 1999; Keely et al., 2000; Andreev et al., 2001), and there are also data suggesting a role of metalloproteinase-mediated cleavage of proHB-EGF (Prenzel et al., 1999).

The aim of the present study was to investigate the role of EGFR transactivation in the signaling mechanisms for GPCR agonists that act as comitogens in rat hepatocytes. We also examined the expression of two early response genes, *c-myc*, which is believed to be required for growth activation (Pelengaris et al., 2002), and *ATF3* ('activating transcription factor 3,' also termed *LRF-1*, 'liver regeneration factor-1'), which is a member of the bZIP superfamily of transcription factors. ATF3 was shown more than a decade ago to be highly expressed in regenerating liver (Hsu et al., 1991), and has more recently been found to have a role in induction of DNA synthesis in mouse hepatoma cells (Allan et al., 2001), and in proliferation and partial transformation in chick embryo fibroblasts (Perez et al., 2001). In the study described here we found that several GPCR agonists, including prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), vasopressin, angiotensin II (Ang.II), and norepinephrine, all of which enhance the effect of EGF on the DNA synthesis, induced the expression of *c-myc* and *ATF3*. Under the same conditions, no effect of any of these agents was observed on EGFR phosphorylation or early downstream responses. Furthermore, neither the induction of *c-myc* and *ATF3*, nor the comitogenic effect or the ERK activity induced by the above GPCR agonists was prevented by the use of an EGFR kinase inhibitor. The results suggest that EGFR transactivation is not required in stimulation of ERK activity, induction of potentially growth-related gene expression, or promotion of DNA synthesis in response to GPCR agonists in hepatocytes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, Dulbecco's phosphate-buffered saline, HEPES, penicillin, and streptomycin were from Gibco (Grand Island, NY). William's medium E was from Whittaker (Walkersville, MD). Norepinephrine hydrogen tartrate, timolol maleate, [Arg^8]vasopressin, Ang.II, $PGF_{2\alpha}$, collagenase (C-0130), dexamethasone, EGF (from mouse), insulin, fatty acid-free bovine serum albumin (BSA) (fraction V), collagen (type I from rat tail), and myelin basic protein (MBP) were obtained from Sigma Chemical Co. (St. Louis, MO). AG1478 was from Calbiochem (La Jolla, CA). [6-^{3}H] thymidine (20–30 Ci/mmol), [γ-^{32}P] adenosine 5′-triphosphate (3,000 Ci/mmol), and [α-^{32}P]dCTP (3,000 Ci/mmol) were from Amersham International (Buckinghamshire, UK). All other chemicals were of analytical quality.

Cell isolation and culture

Male Wistar rats, 170–230 g, were fed ad libitum. Hepatocytes were isolated by a two-step in vitro version (Seglen, 1976) of the collagenase perfusion technique (Berry and Friend, 1969) with modifications as previously described (Christoffersen et al., 1984). The hepatocytes were seeded onto Costar plastic culture wells or flasks, at a cell density of $2 \times 10^4/cm^2$. The culture medium was a serum-free 1:1 combination of William's medium E and Dulbecco's modified Eagle's medium (with final glucose concentration 8.4 mM). The medium was supplemented with penicillin (67 μg/ml), streptomycin (100 μg/ml), collagen (3 μg/ml), insulin (100 nM), and dexamethasone (25 nM). The cultures were kept in 95% air/5% CO_2 at 37°C.

Stimulation of hepatocytes and pre-treatment with the EGFR tyrosine kinase inhibitor AG1478

For estimation of the comitogenic effect of the GPCR agonists, cells were cultured for 3 h before stimulation with GPCR agonists as indicated. In these experiments EGF (5 nM) and [^{3}H] thymidine (1 μCi/ml, 0.125 Ci/mmol) were added to the cultures 24 h after plating. In studies of the mitogenic effect of EGF alone, hepatocytes were grown for 24 h prior to addition of EGF and [^{3}H] thymidine. In both cases, cells were harvested after 50 h. In studies of the effect of GPCR agonists on DNA synthesis, DMSO (0.5%) or AG1478 (5 μM) was added 3 h after plating, and the cells stimulated 30 min later. Due to rapid degradation of AG1478, cells were treated with AG1478 (5 μM) a second time, 5 h after the first addition (maximum DMSO conc. 1%). When cells were stimulated with EGF alone an identical pre-treatment with DMSO or AG1478 was performed 30 min before stimulation (24 h after plating), and the inhibitor was added again 5 h after the first addition of AG1478 (maximum DMSO conc. 1%). Hepatocytes prepared for Northern blot analysis were cultured for 4.5 h, pre-treated with DMSO (0.5%) or AG1478 (5 μM) for 30 min, and then stimulated for 1 h with GPCR agonists or EGF as indicated.

Immunoblotting

Hepatocytes were exposed to GPCR agonists for the indicated time periods after 3 h of culturing. Aliquots with 20 μg cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed on 10% (w/v) polyacrylamide gels (acrylamide:N′N′-bis-methylene acrylamide 30:0.8) followed by immunoblotting with a phospho-specific MAP kinase antibody detecting $p44^{mapk}$ and $p42^{mapk}$ (ERK1 and 2) only when catalytically activated by phosphorylation at Tyr204 or Thr202/Tyr204, (New England Biolabs, Inc., Beverly, MA). Immunoreactive bands were visualized with Bio West Extended Duration Chemiluminescent Substrate (UVP, Inc., Upland, CA).

Immunoprecipitation (IP)

Analysis of EGFR phosphorylation was based on a method described previously (Yamashita et al., 1998). After agonist stimulation 3 h after plating, the cells were rapidly rinsed twice in ice cold 0.9% NaCl, and once in IP buffer A (50 mM Tris-HCl, pH 7.4, 280 mM NaCl, 0.2 mM EDTA, 2.0 mM EGTA, 10% glycerol, 1 mM activated Na_3VO_4, 50 mM $Na_4P_2O_7$, 1 mM phenylmetylsulfonyl fluoride (PMSF), and Complete protease inhibitor (Boehringer Mannheim, Germany)). Cells were lysed in 1 ml IP buffer A, supplied with Nonidet P-40 (NP-40) to a final concentration of 1%, for 15 min on ice and the lysates were transferred into Eppendorf tubes. Insoluble material was pelleted at 13,000 rpm for 10 min at 4°C. Clarified lysates (approximately 250 μg protein) were incubated for 60 min with polyclonal EGFR antibody (SC-03, Santa Cruz Biotechnology, Santa Cruz, CA) and the complexes were captured with Dynabeads® (Dynal AS, Oslo, Norway), coated with sheep anti-rabbit antibodies (a gift from Dr. Kjell Nustad, The Norwegian Radium Hospital, Oslo, Norway). Complexes were washed with IP buffer A with 0.5% NP-40.

Shc and Pyk2 proteins were immunoprecipitated under denaturating conditions. After agonist stimulation 3 h after plating, the cells were lysed inn 500 μl boiling lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM Na_3VO_4), then the lysates were boiled for 5 min. After five passages through a 27× G gauge on ice, the lysates were cleared by centrifugation (10 min, 13,000 rpm at 4°C). 250 μg (Shc analysis) or 500 μg (Pyk2 analysis) protein was incubated 2 h in IP-buffer B (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5% NP-40, and 1% Triton-X100) with phosphotyrosine antibody (PY-99, Santa Cruz Biotechnology). The immune complexes were captured with Dynabeads®, coated with sheep anti-mouse IgG. Complexes were washed with ice-cold IP-buffer B.

The proteins were released from the Dynabeads® by boiling for 5 min in 2× Laemmli sample buffer. Immunoprecipitates were separated in 8–12% SDS–PAGE gels as described, depending on protein weight, and transferred to nitrocellulose membranes. The membranes were either blocked in T-TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 5% low fat drymilk and 0.05% Tween-20, or T-TBS with 1% BSA for detection with RC-20: direct horseradish peroxidase (HRPO) conjugated phosphotyrosine antibody. Membranes were then incubated overnight at 4°C with appropriate primary antibodies, RC-20 phosphotyrosine antibody, monoclonal Pyk2 antibody (both from Transduction Laboratories, Lexington, KY), 4g10 phosphotyrosine antibody, or polyclonal anti-Shc (both from Upstate Biotechnology, Lake Placid, NY). Membranes were further incubated with secondary antibody, HRPO conjugated sheep anti-rabbit or sheep anti-mouse, depending on the primary antibody, in blocking solution for 1.5 h. The blots were visualized with Enzyme-linked Chemiluminesence Reagent (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), or Bio West Extended Duration Chemiluminescent Substrate (UVP, Inc.).

Measurement of ERK activity

The measurement of ERK1/2 activity was performed as previously described (Anderson et al., 1991; Melien et al., 1998; Thoresen et al., 1998). In brief, the hepatocyte cultures were exposed to agonists for 5 min before rinsing and scraping the cells into a 10% ethylene glycol buffer. The lysate was precleared by centrifugation (16,000*g*) for 10 min, and the supernatant was mixed with phenyl-Sepharose which was washed twice in a 10%, twice in a 35% ethylene glycol buffer, and finally ERK1/2 was eluted with a 60% ethylene glycol buffer. The eluate was assayed for ERK1/2 activity with MBP as substrate, thereafter spotted onto P81 paper (Whatman, Maidstone, UK), which was washed, dried, and counted in a liquid scintillation counter.

RNA extraction and Northern blot analysis

RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction according to the procedure described by Chomczynski and Sacchi (1987) and modified by Houge et al. (1990). Ten microgram of total RNA was electrophoresed in 1.5% agarose gel in the presence of 6.6% formaldehyde. The RNA was transferred to Biotrans nylon membranes, UV exposed for 1 min and baked for 1 h at 80°C. The filters were prehybridized overnight in 50% formamide, 5× Denhardt's solution, 6× SSC, and 1% SDS at 42°C, and hybridized overnight in the same solution. The probes [a 1.9 kb *Eco*R1 fragment of rat ATF3/LRF-1 (Hsu et al., 1991) and a 1.4 kb *Bam*HI fragment of mouse c-myc cDNA (Land et al., 1983)] was ^{32}P-dCTP labeled by random priming (Rediprime II DNA Labeling System, Amersham Biosciences, Buckinghamshire, UK). After hybridization the filters were washed 4-times in 2× SSC/0.1% SDS at 25°C for 5 min and twice in 0.1× SSC/0.1% SDS at 50°C for 30 min. The hybridized bands were visualized and analyzed by Instant Imager Electronic Autoradiography (Packard Instrument Company, Meridan, CT), and by autoradiography.

Other methods

DNA synthesis was measured by determining the amount of [^{3}H] thymidine incorporated into DNA (Refsnes et al., 1994). Protein was determined with the BCA Protein Assay (Pierce, Rockford, IL).

RESULTS

GPCR agonists, unlike EGF, activate Pyk2

A role for Pyk2, through interaction with c-Src, has been suggested in the EGFR transactivation pathway

from GPCRs (Eguchi et al., 1999; Keely et al., 2000; Andreev et al., 2001). IP of tyrosine phosphorylated proteins showed that Ang.II, vasopressin, $PGF_{2\alpha}$, and norepinephrine induced a rapid increase in tyrosine phosphorylation of Pyk2. As demonstrated with vasopressin, the response was sustained for at least 30 min (Fig. 1A). Treatment of cells with Ang.II, $PGF_{2\alpha}$, or norepinephrine resulted in similar time courses (data not shown); phosphorylation after 5 min of stimulation is demonstrated in Figure 1B. In contrast, 5 min stimulation with EGF did not induce any increase in the level of Pyk2 phosphorylation (Fig. 1B). Although the precise role of Pyk2 has not been examined, these novel results suggest that activated Pyk2 might be a part of the pathways downstream of GPCRs in hepatocytes.

EGFR is not phosphorylated after stimulation with GPCR agonists

To search for evidence of transactivation, we measured EGFR tyrosine phosphorylation after IP in cells exposed to Ang.II, vasopressin, PGF2α, or norepinephrine. Since ERK activity induced by these agents in rat hepatocytes is known to peak within 3–5 min (Melien et al., 1998), any EGFR transactivation involved in the stimulation of ERK was expected to appear rapidly. However, as shown in Figure 2A, after stimulation for 1 or 5 min, none of the GPCR agonists tested induced any increase in the level of EGFR phosphorylation, compared to basal. EGFR phosphorylation levels were also determined in cultures stimulated for several hours. As shown in Figure 2B, there was no evidence of increased phosphorylation levels after stimulation with norepinephrine for up to 5 h. Similar results were obtained in hepatocytes stimulated with Ang.II (data not shown). Thus, although the above results suggest a possible role for Pyk2 in signaling from GPCRs in rat hepatocytes, increase in the phosphorylation status of the EGFR does not appear to precede the ERK activity induced by norepinephrine, vasopressin, Ang.II, or $PGF_{2\alpha}$, or take place up to 5 h after addition of agonist.

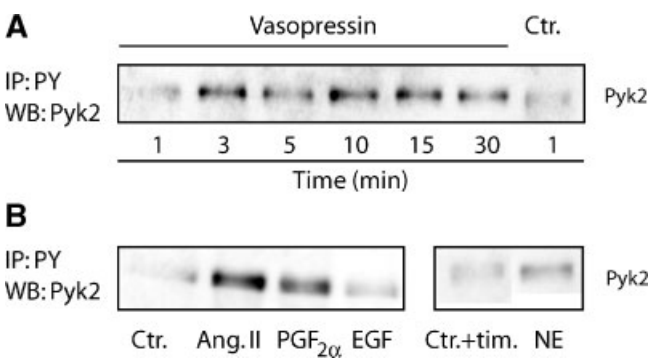

Fig. 1. Activation of Pyk2 by G protein-coupled receptor (GPCR) agonists. Pyk2 activity was detected by immunoprecipitation (IP) with a phosphotyrosine antibody and immunoblot analysis with an antibody against Pyk2. **A**: Hepatocytes were cultured for 3 h before stimulation with 1 μM vasopressin. Results represent one typical experiment. **B**: Hepatocytes were cultured for 3 h before stimulation for 5 min with 1 μM angiotensin II (Ang.II), 100 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 100 μM norepinephrine (NE) (in the presence of timolol), or 10 nM epidermal growth factor (EGF). Results represent one typical experiment.

Shc is not activated by GPCR agonists in cultured rat hepatocytes

The adaptor protein Shc is known to be tyrosine-phosphorylated by activation of many different receptors, among them RTKs and GPCRs (Ravichandran, 2001). We used Shc phosphorylation as an alternative approach to detect EGFR involvement in signaling from GPCR in rat hepatocytes. The hepatocytes were stimulated with GPCR agonists for up to 60 min prior to IP of tyrosine-phosphorylated proteins and detection of Shc after SDS–PAGE. As demonstrated with norepinephrine and $PGF_{2\alpha}$ (Fig. 3), the experiments provided no evidence of increased Shc phosphorylation. Experiments with vasopressin and Ang.II gave similar results (data not shown). In contrast, EGF induced significant phosphorylation of two Shc isoforms (p46 and p52). These results did not support the idea of EGFR transactivation being involved in short time effects of GPCR agonists.

Activation of ERK in response to GPCR agonists is not impaired by the EGFR tyrosine kinase inhibitor AG1478

To further examine the role of the EGFR in GPCR-mediated mechanisms, we tested the effect of AG1478, a specific EGFR tyrosine kinase inhibitor, on ERK activity induced by GPCR agonists. As shown in Figure 4A, a 30 min preincubation with 5 μM AG1478 completely abolished phosphorylation of ERK1/2 subsequent to stimulation with EGF. Next, the effect of 5 μM AG1478 on ERK activity induced by ligands acting on GPCRs was tested in an ERK activity assay with MBP as substrate. The data obtained showed that ERK activity induced by vasopressin, Ang.II, $PGF_{2\alpha}$, or norepinephrine, was not reduced in hepatocytes pretreated with 5 μM AG1478. In contrast, AG1478 reduced the EGF stimulated ERK activity to basal level (Fig. 4B). The effect of AG1478 on activation of ERK was confirmed in Western blots with an antibody against double phosphorylated ERK1/2 (Fig. 4C).

Comitogenic effect of GPCR agonists is not prevented by AG1478

Because comitogenic effects in cultured rat hepatocytes are exerted primarily in the early G_1 phase (0–10 h after plating) and the cells become responsive to the mitogenic effect of EGF in mid/late G_1 (Christoffersen et al., 2000), we examined the role of EGFR function in GPCR-mediated growth stimulation by treating the cells with AG1478 in early G_1. These experiments necessitate careful control of dose and timing, since they depend on EGFR inhibition for 5–10 h after administration of the GPCR agonist but also require that EGFR functionality is fully restored prior to the subsequent addition of EGF in mid-G_1 (24 h in these experiments). As demonstrated above, AG1478 inhibited EGF-induced ERK phosphorylation completely at 5 μM (Fig. 4A), however, the effect of AG1478 was gradually reversed within approximately 4 h (data not shown), and readdition of the inhibitor after 5 h (8 h after plating) was necessary. Experiments showed that when the hepatocytes were treated with AG1478 twice at 24 and 29 h, as illustrated in the protocol scheme in

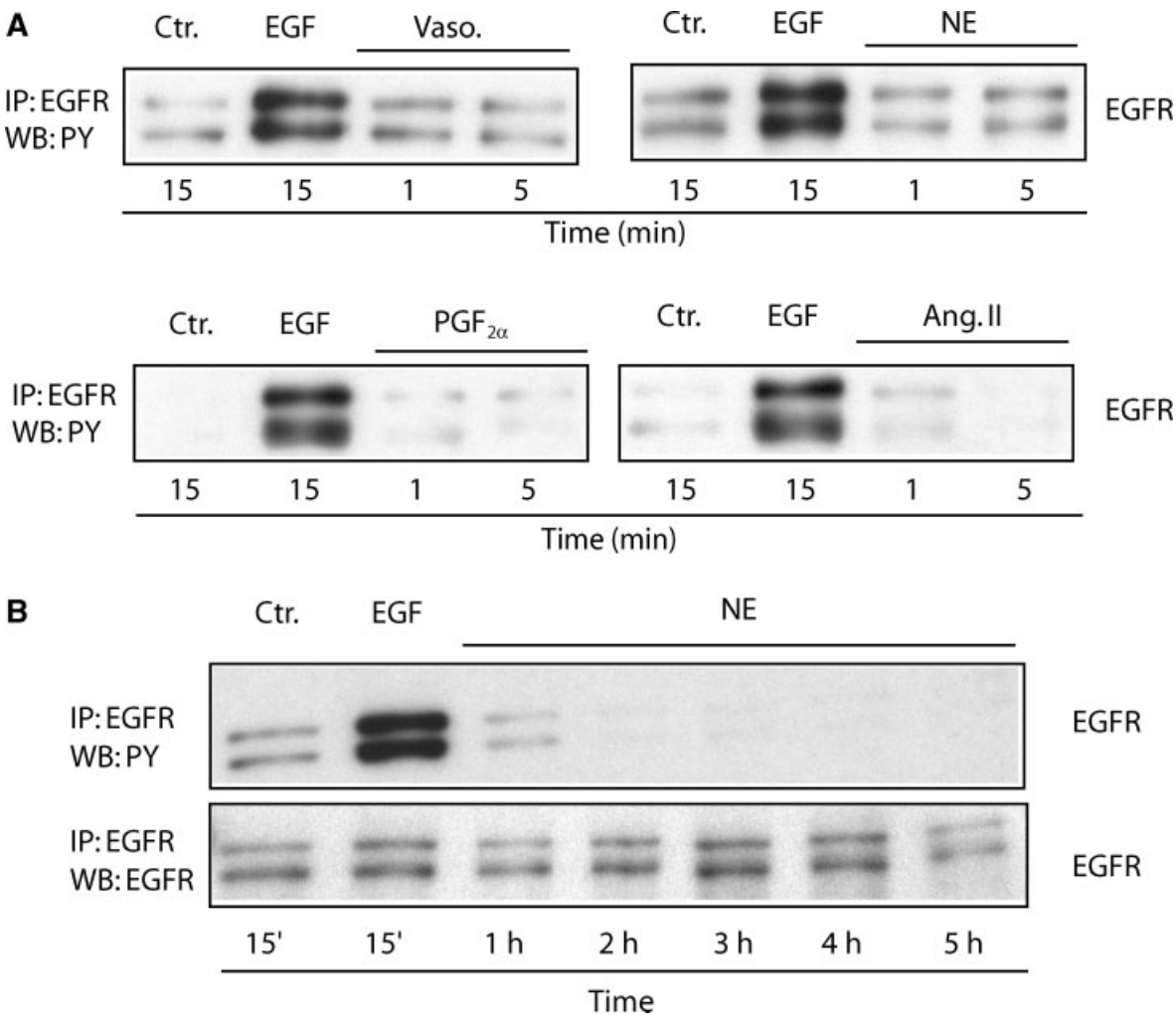

Fig. 2. The EGF receptor (EGFR) is not phosphorylated upon stimulation with GPCR agonists. EGFR phosphorylation was detected by IP with an EGFR antibody and immunoblot analysis with a phosphotyrosine antibody. Hepatocytes were cultured for 3 h before stimulation with 100 μM norepinephrine (NE) (in the presence of timolol), 1 μM vasopressin (Vaso.), 1 μM Ang.II, 100 μM $PGF_{2\alpha}$, or 10 nM EGF, as indicated. Results represent one typical experiment.

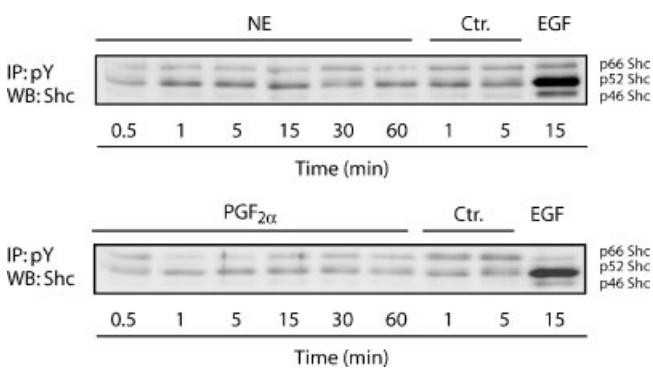

Fig. 3. Shc is not activated by GPCR agonists. Shc activity was detected by IP with a phosphotyrosine antibody and immunoblot analysis with an antibody recognizing the 46, 52, and 66 kDa Shc proteins. Hepatocytes were cultured for 3 h before stimulation with 100 μM norepinephrine (NE) (in the presence of timolol), 100 μM $PGF_{2\alpha}$, or 10 nM EGF. Results represent one typical experiment.

Figure 5A, the mitogenic effect of EGF was completely inhibited (Fig. 5B).

Treatment of the cells with AG1478 early in G_1 did not suppress the comitogenic effects of the GPCR agonists (Fig. 6B). However, a slight reduction of the comitogenic effect was noted in some of the experiments conducted with norepinephrine (Fig. 6B). We have no clear explanation for this observation. One possibility is that different GPCR agonists might exert their comitogenic effects through partially different pathways, and that signaling from α_1-adrenergic receptors is different than signaling from the other GPCRs. On the other hand, more detailed studies on residual effects of AG1478 suggest that in some cases, a partial inhibition of EGFR by AG1478 can persist until the time of EGF administration (data not shown). However, no reduction in EGF-induced ERK activity was seen in cells treated by the same protocol (Fig. 6C). One possible explanation for the contradiction could be that ERK as detected by

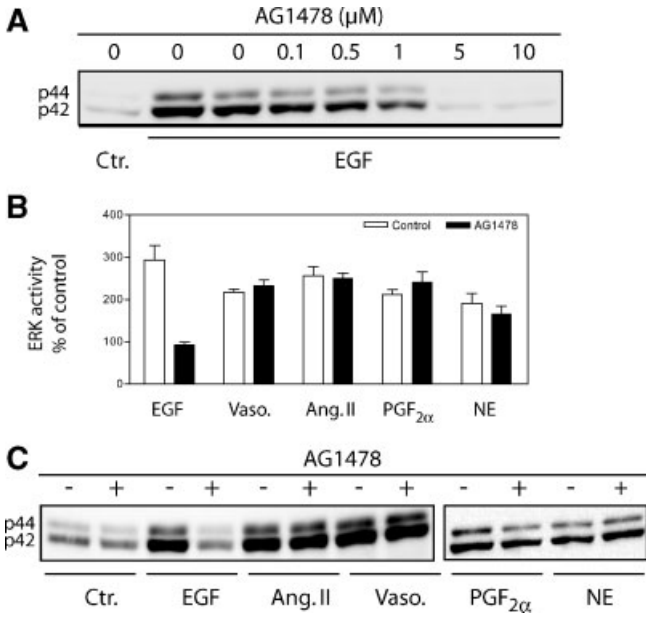

Fig. 4. Extracellular signal-regulated kinase (ERK) activity induced by GPCR agonists is not impaired by the selective EGFR tyrosine kinase inhibitor AG1478. **A**: ERK kinase activity was detected by immunoblot analysis with an antibody against phosphorylated ERK1/2. Hepatocytes were cultured for 2.5 h and pre-treated with DMSO (0.5%), or AG1478 for 30 min before stimulation for 5 min with 10 nM EGF. Results represent one typical experiment. **B**: ERK kinase activity was detected in an in vitro kinase assay as described in "Materials and Methods." Hepatocytes were cultured for 2.5 h and pre-treated with DMSO (0.5%) or AG1478 (5 μM) for 30 min before stimulation for 5 min with 10 nM EGF, 1 μM vasopressin (Vaso.), 1 μM Ang.II, 100 μM $PGF_{2\alpha}$, or 50 μM norepinephrine (NE) (in the presence of timolol). Results represent mean ± SEM of two separate experiments, each with duplicates. **C**: ERK kinase activity was detected by immunoblot analysis with an antibody against phosphorylated ERK1/2. Hepatocytes were cultured for 2.5 h and pre-treated with DMSO (0.5%) or AG1478 (5 μM) for 30 min before stimulation for 5 min with 10 nM EGF, 1 μM Ang.II, 1 μM vasopressin (Vaso.), 100 μM $PGF_{2\alpha}$, or 50 μM norepinephrine (NE) (in the presence of timolol). Results represent one typical experiment.

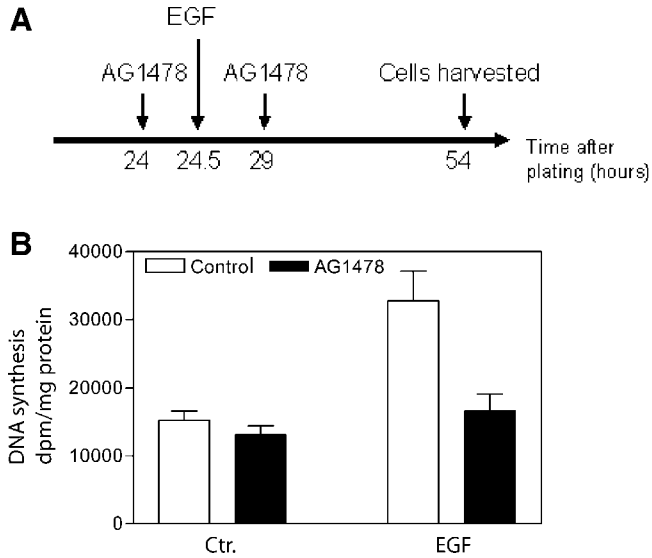

Fig. 5. EGF-induced DNA synthesis is completely abolished by the EGFR tyrosine kinase inhibitor AG1478. **A**: Protocol scheme illustrating time points after plating for addition of AG1478 and EGF, as well as harvesting of the cells. **B**: DNA synthesis was assessed as described in "Materials and Methods." Hepatocytes were cultured for 24 h and pre-treated for 30 min with DMSO (0.5%), or AG1478 (5 μM) prior to addition of 10 nM EGF and [^{3}H] thymidine. DMSO (0.5%), or AG1478 (5 μM) was added a second time 29 h after plating and cells were harvested at 54 h after plating. Results represent mean ± SEM of three separate experiments.

immunoblotting is less sensitive than DNA synthesis to detect a small residual effect of AG1478. Taken together, the results are compatible with comitogenic mechanisms that are independent of EGFR transactivation.

GPCR agonists induce the expression of mRNAs for c-myc and ATF3 independently of EGFR function

It is likely that growth-promoting pathways from GPCRs activate a set of critical early response genes, and it is of interest to identify such genes and to decide whether or not their expression is induced through mechanisms of EGFR transactivation. It has been found that certain GPCR agonists increase the expression of the proto-oncogenes c-*fos*, c-*myc*, and c-*mos* in isolated rat hepatocytes (Gonzalez-Espinosa and Garcia-Sainz, 1992). For the present studies we have chosen *c-myc*, which seems to be required for the growth response (Pelengaris et al., 2002), and *ATF3*, which has been implicated in growth-stimulatory mechanisms in certain cells and whose expression is increased in regenerating liver (Weir et al., 1994).

Northern analysis demonstrated increased expression of c-myc mRNA in hepatocytes stimulated for 1 h with vasopressin, Ang.II, norepinephrine, and $PGF_{2\alpha}$ (Fig. 7B). Furthermore, markedly increased levels of mRNA for ATF3 were observed in hepatocytes stimulated with the same agonists, which is a novel finding (Fig. 7C). The response decreased to near control levels within 4 h after stimulation, as shown for vasopressin in Figure 7A. The effect of inhibiting EGFR kinase activity was examined, using the EGFR kinase inhibitor AG1478. As shown in Figure 7B,C, there was no significant reduction in the GPCR agonist-induced expression of mRNA for c-myc or ATF3 when cells were pre-treated with AG1478 (5 μM). In contrast, the increase in mRNA for ATF3 and c-myc induced by EGF was totally abolished in the presence of AG1478. Examples of typical Northern blots for c-myc and ATF3 in hepatocytes stimulated with $PGF_{2\alpha}$, Ang.II, or EGF, in the presence or absence of AG1478, are given in Figure 7D,E, respectively.

DISCUSSION

The results of the present study suggest that signaling from GPCRs in rat hepatocytes, such as stimulation of ERK activity, induction of growth-related gene expression, and comitogenic effects do not require EGFR transactivation.

Previous studies have shown that vasopressin, norepinephrine, Ang.II, and $PGF_{2\alpha}$ activate ERK through pathways that depend on calcium and PKC and that their effects are diminished by inhibitors of the Src kinase family (Dajani et al., 1999; Melien et al., 2002).

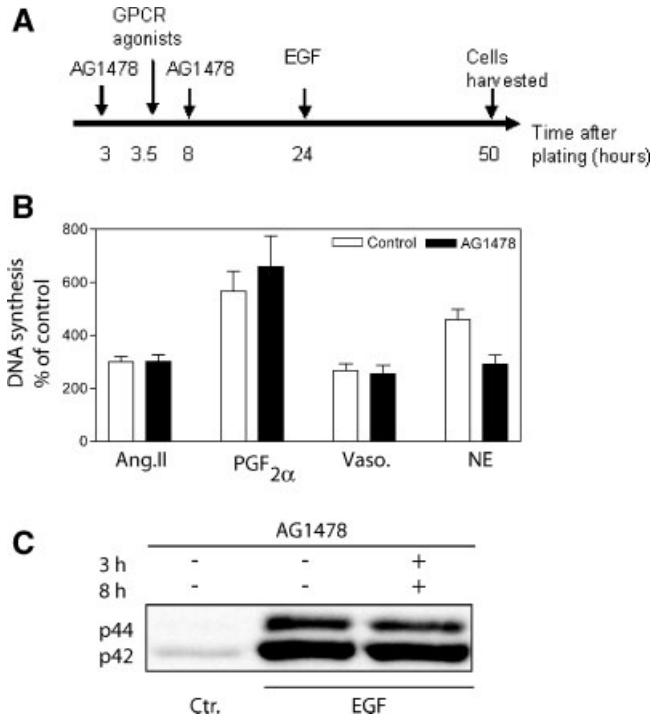

Fig. 6. The comitogenic effect of GPCR agonists is largely not affected by the EGFR tyrosine kinase inhibitor AG1478. **A**: Protocol scheme illustrating time points after plating for addition of AG1478, GPCR agonists, and EGF as well as harvesting of the cells. **B**: DNA synthesis was assessed as described in "Materials and Methods." Hepatocytes were cultured for 3 h and pre-treated for 30 min with DMSO (0.5%), or AG1478 (5 μM) prior to addition of 1 μM Ang.II, 100 μM $PGF_{2\alpha}$, 1 μM vasopressin (Vaso.), or 50 μM norepinephrine (NE) (in the presence of timolol). DMSO (0.5%), or AG1478 (5 μM) was added a second time 8 h after plating, and 5 nM EGF and [^{3}H] thymidine were added at 24 h. Cells were harvested 50 h after plating. Results represent mean ± SEM of six to seven separate experiments. **C**: ERK kinase activity was detected by immunoblot analysis with an antibody against phosphorylated ERK1/2. Hepatocytes were treated with DMSO (0.5%) or AG1478 (5 μM) at 3 and 8 h after plating, and stimulated for 5 min with 10 nM EGF at 24 h after plating. Results represent one typical experiment.

All these mechanisms have been implicated in transactivation of the EGFR by GPCR agonists (Eguchi et al., 1998, 1999; Luttrell et al., 1999; Keely et al., 2000; Andreev et al., 2001). In the present study we have examined the role of EGFR transactivation in hepatocytes, using different cellular responses as endpoints. Our results indicate that EGFR phosphorylation was not increased after stimulation with GPCR agonists, neither was EGFR activity, in the form of Shc phosphorylation, observed. Since Shc phosphorylation is also involved in signaling from other RTKs, the data may have the broader implication that they are not transactivated either. Furthermore, there was no evidence for a role of the EGFR in effects like stimulation of ERK activity, or early gene expression. Furthermore, the enhanced DNA synthesis in response to GPCR agonists was largely unaffected by AG1478. These results, taken together, suggest that immediate transactivation of the EGFR is not required for the comitogenic effects of these agents. However, we cannot exclude the possibility that stimulation with vasopressin, Ang.II, $PGF_{2\alpha}$, or norepinephrine might lead to increased production or release of an autocrine growth factor. For example, expression and secretion of the EGFR ligand transforming growth factor-α (TGF-α) is reported to increase during liver regeneration (maximal mRNA levels 24 h after hepatectomy), as well as within days after stimulation of cultured hepatic cells with growth factors (Mead and Fausto, 1989; Shiota et al., 1994). Although accumulating evidence indicates the importance of receptor crosstalk, it is increasingly recognized that the contribution of EGFR transactivation to GPCR agonist-induced responses, such as ERK activation and cell proliferation, depends on cell type. In Rat-1 cells, bombesin induced ERK activation via an EGFR-dependent mechanism, whereas the same agonist promoted EGFR-independent ERK activation in Swiss 3T3 cells. Interestingly, the mitogenic effect of bombesin in Swiss 3T3 cells was dependent on EGFR function (Santiskulvong et al., 2001). In GN4 rat liver epithelial cells, Ang.II has been found to stimulate ERK via two independent pathways; a putative Ras/Raf-independent, PKC-dependent pathway, and an EGFR/Ras/Raf-dependent pathway observed when PKC activation is prevented (Li et al., 1998).

The mechanisms involved in the comitogenic effect of GPCR agonists in hepatocytes are incompletely understood. In previous studies we found evidence that sustained accumulation of diacylglycerol (DAG), with activation of PKC, is involved in the growth-promoting effects of vasopressin, Ang.II, and norepinephrine (Dajani et al., 1999). Furthermore, while ERK activity is essential for the mitogenic effect of EGF in hepatocytes (Talarmin et al., 1999; Thoresen et al., 2003), we recently found that the comitogenic effect of vasopressin, norepinephrine, Ang.II, and $PGF_{2\alpha}$ is independent of ERK activity (Nilssen et al., 2002). In vascular smooth muscle cells, a role for Pyk2 in increased expression of c-fos induced by Ang.II has been proposed (Rocic et al., 2002), suggesting that this calcium-sensitive tyrosine kinase might be a mediator in the growth-promoting effects of calcium-mobilizing agents. Our results demonstrate evidence of GPCR agonist-induced Pyk2 activity under circumstances where transactivation does not appear to take place. Thus, it is possible that Pyk2 has a different role in signaling from GPCRs in hepatocytes, although at present we do not have any conclusive data on this topic.

The presence of growth-promoting humoral factors during G_1 results in a number of cellular events, including gene expression and phosphorylation of proteins involved in early cell cycle progression (Sherr, 1996). We examined the expression of the two early response genes *c-myc* and *ATF3* in cells exposed to EGF, vasopressin, Ang.II, $PGF_{2\alpha}$, or norepinephrine. ATF3 mRNA is barely detectable in normal rat liver, but is dramatically induced upon hepatectomy (Weir et al., 1994). For instance, ATF3 has been shown to induce DNA synthesis in mouse hepatoma cells and proliferation in chick embryo fibroblasts (Allan et al., 2001; Perez et al., 2001), and ATF3 antisense oligonucleotide is reported to exert inhibitory effect on ectopic growth of human colon cancer cells (Ishiguro et al., 2000). Furthermore, rapid induction of mRNA for ATF3, jun-B, c-fos as well as

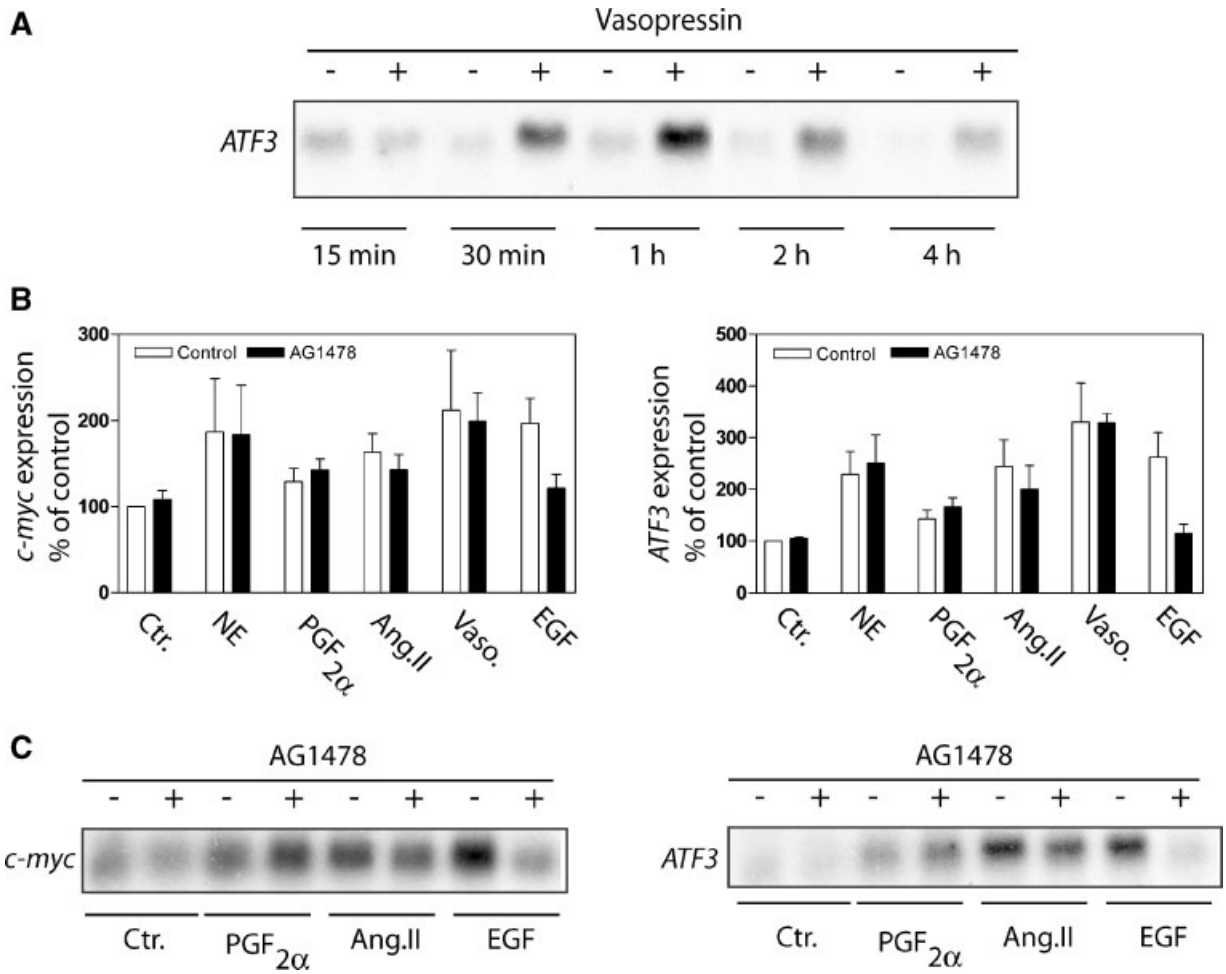

Fig. 7. GPCR agonist-induced increase in expression of mRNAs for activating transcription factor 3 (ATF3) and c-myc is not impaired by inhibition of EGFR function. Hepatocytes were cultured for 4.5 h prior to stimulation with GPCR agonists. In some experiments 30 min pre-treatment with DMSO (0.5%) or AG1478 (5 μM) was performed before cells were stimulated with 50 μM norepinephrine (NE) (in the presence of timolol), 100 μM $PGF_{2\alpha}$, 1 μM Ang.II, 1 μM vasopressin (Vaso.), or 10 nM EGF for 60 min. RNA extraction and Northern blot analysis was performed as described in "Materials and Methods." **A**: Time course for vasopressin-induced expression of mRNA for ATF3. Results represent one typical experiment. **B**: Effect of GPCR agonists and EGF, in the presence and absence of AG1478, on the level of c-myc mRNA. **C**: Effect of GPCR agonists and EGF, in the presence and absence of AG1478, on the level of ATF3 mRNA. Results represent mean ± SEM of two to three experiments with GPCR agonists, and of five experiments with EGF. **D**: A typical Northern blot showing the effect of Ang.II, $PGF_{2\alpha}$, and EGF, in the presence and absence of AG1478, on the level of c-myc mRNA. **E**: A typical Northern blot showing the effect of Ang.II, $PGF_{2\alpha}$, and EGF, in the presence and absence of AG1478, on the level of ATF3 mRNA.

c-myc protein has been reported in cultured hepatocytes stimulated with various growth factors or hormonal agents (Weir et al., 1994; Skouteris and Schroder, 1996). Ang.II, vasopressin, epinephrine, and the phorbol ester PMA have been shown to increase the expression of the proto-oncogenes *c-fos*, *c-myc*, and *c-mos* in isolated rat hepatocytes (Gonzalez-Espinosa and Garcia-Sainz, 1992). Thus, a possible mechanism involved in the comitogenic effect of GPCR agonists might be expression of genes necessary for progression of hepatocytes through the cell cycle.

In agreement with previous studies, our results showed that expression of both genes was enhanced in cells treated with EGF (Skouteris and Kaser, 1991; Weir et al., 1994). All the GPCR agonists also induced increased expression of mRNAs for c-myc. In addition we made the novel observation of marked increase in expression of ATF3 mRNA in response to GPCR agonists. Thus, a role for these genes in the growth promoting effects of the comitogenic agents is conceivable. The present results also suggest that this gene expression is exerted independently of EGFR transactivation.

In conclusion, the results of the present study demonstrate increased levels of ATF3 mRNA in hepatocytes in response to activation of GPCRs, introducing early gene expression as a possible mechanism in the comitogenic effect of these agents. Furthermore, EGFR transactivation is not required in signaling pathways from GPCRs in hepatocytes, such as increased ERK activity, induction of potentially growth-related gene expression, and most likely DNA synthesis.

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