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The role of ErbB3/HER3 in gliomas and breast cancer: Molecular mechanisms and potential role as therapeutic target

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Erklärung

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Ehrenwörtliche Versicherung

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1. Summary

The two members of the epidermal growth factor receptor (EGFR) family, ErbB2/HER2 and ErbB3/HER3, act as key oncogenes in cancer cells. ErbB3/HER3 is a receptor tyrosine kinase (RTK) believed to have an impaired kinase activity due to two uncharacteristic amino acid residues in the tyrosine kinase consensus sequences. Heregulin (HRG), a specific ligand for ErbB3/HER3 promotes the heterodimerization with the RTK ErbB2/HER2 and transphosphorylation of its intracellular domain. ErbB3/HER3 preferentially forms heterodimers with ErbB2/HER2, which induce the most potent mitogenic signal among EGFR family members.

We investigated the HRG-dependent mechanism by which the cytoplasmic proline-rich tyrosine kinase PYK2 becomes tyrosine phosphorylated, its role in the MAPK pathway and subsequent effects on the invasive properties of tumour cells. The results presented in this thesis show that ErbB3/HER3 is able to phosphorylate PYK2 upon stimulation with HRG. In glioma-derived cell lines PYK2 is constitutively associated with ErbB3/HER3 but not ErbB2/HER2 and stimulation with HRG results in PYK2 tyrosine phosphorylation requiring active kinase functions of both ErbB2/HER2 and ErbB3/HER3. Our data suggest a pivotal role for PYK2 as a regulator of the invasive capacity of glioma cells and, furthermore, show that ErbB3/HER3 is a functional RTK with narrow substrate specificity.

Moreover, this study also aimed at determining whether ErbB3/HER3 plays, beside ErbB2/HER2, a physiologically relevant role in transmitting proliferative and migratory signals in breast cancer cell lines. An agonistic monoclonal antibody against ErbB2/HER2, Herceptin®, has previously been shown to interfere with ErbB2/HER2 signalling and is therapeutically effective in humans. Therefore, we dissected the effects of an antibody against ErbB3/HER3 (α-HER3^{ECD}) and Herceptin® on HRG-mediated

signalling and analyzed the inhibitory potential of α-HER3^{ECD} on breast cancer cell growth and motility.

These data indicate that this antibody is a more potent inhibitor than Herceptin® for the HRG-mediated signal transduction processes. Furthermore, our results reinforce the notion that ErbB3/HER3 could be a key target in cancer drug designing and show the great potential of anti-ErbB3/HER3 antibodies for the therapy of breast cancer and other malignancies characterized by the overexpression of ErbB3/HER3.

2. Introduction

2.1. The epidermal growth factor receptor family

2.1.1. ErbB1/EGFR-the "godfather" of the EGFR family

ErbBs are typical receptor tyrosine kinases that were implicated in cancer in the early 1980s when the avian erythroblastosis tumour virus was found to encode an aberrant form of the human epidermal growth factor receptor (also known as Erb1, HER1 or EGFR) (Downward et al., 1984b). The four ErbBs share an overall structure of two cysteine-rich stretches in their extracellular region, and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites (Downward et al., 1984a; Riedel et al., 1987) (Fig. 1).

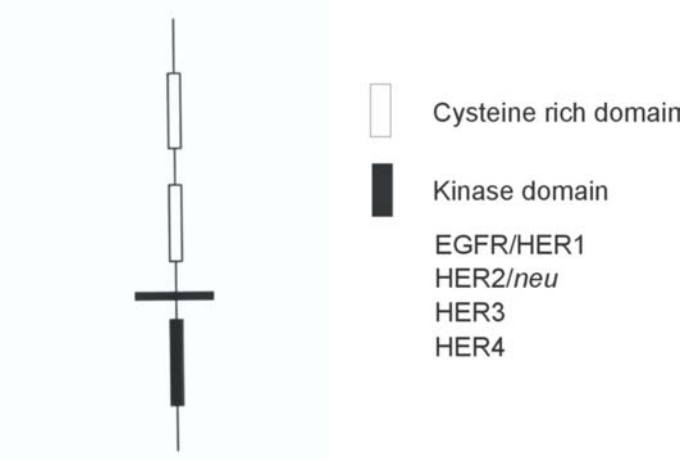

Fig.1: **Schematic representation of EGFR family RTKs**

With few exceptions (for example, haematopoietic cells), ErbB proteins are expressed in cells of mesodermal and ectodermal origins (Yarden and Sliwkowski, 2001). The EGFR was the first signal-generating protein and protooncogene with known normal function to be cloned molecularly (Ullrich et al., 1984). Several lines of evidence indicate a prominent role of the EGFR in cancer: its functional role as a protooncogene in viruses, the pathophysiological effect of EGFR mutants and its overexpression in several cancer types are well documented (Gill, 1989). Furthermore, its

overexpression correlates inversely with estrogen receptor (ER) status, is often detected in invasive ductal carcinomas and is associated with poor prognosis.

2.1.2. ErbB2/HER2-an essential breast cancer oncogene

A member of the EGFR family, ErbB2/HER2 (known as *neu*) has been identified as one of the key oncogenes in invasive breast cancer (Natali et al., 1990; Schechter et al., 1985; Slamon et al., 1989). ErbB2/HER2 gene amplification is found in 20-30% of all diagnosed breast cancer patients and a significant correlation between ErbB2/HER2 overexpression and reduced survival of breast cancer patients has been found (Slamon et al., 1989). Therefore, ErbB2/HER2 became a target in breast cancer therapy due to its signalling properties in breast cancer. It is important in breast cancer therapy also from the prognostic point of view. Moreover, ErbB2 is not only a prognostic factor but also a predictive marker for responses to various therapeutic agents used in breast cancer therapy (Ross and Fletcher, 1998) (Tsongalis and Ried, 2001). ErbB2/HER2 overexpression correlates with a lack of response to endocrine therapy and chemotherapeutic agents.

2.1.3. ErbB3/HER3-the "neglected" receptor of the EGFR family

Another member of the EGFR family, HER3 (ErbB3), is consistently overexpressed together with ErbB2/HER2 in invasive breast cancer and a correlation between the expression of ErbB2/HER2-ErbB3/HER3 and the progression from a non-invasive to an invasive stage has been shown (Alimandi et al., 1995; deFazio et al., 2000; Naidu et al., 1998). ErbB3/HER3 is a receptor tyrosine kinase, which was reported to have a weak activity (Guy et al., 1994); it forms with ErbB2/HER2 a high-affinity complex for its natural ligand HRG (Sliwkowski et al., 1994). ErbB3 is expressed in several cancers, but there is no evidence for gene amplification and overexpression is limited. However, a recent multinational study found that co-expression of ErbB2/HER2 with EGFR or ErbB3/HER3 in oral squamous-cell carcinoma was significant and it critically improved the predicting power (Xia et al., 1999). Therefore, the role of ErbB3/HER3 as a prognostic marker could become more important at least in oral squamous-cell carcinoma. Similarly, analysis of prostate cancer suggests the existence of a paracrine loop involving Neuregulin 1 (NRG1) and the ErbB2/HER2–ErbB3/HER3 heterodimer (Lyne et al., 1997).

2.2. The NRGs-Ligands of the ErbB family

All high-affinity ErbB ligands have an EGF-like domains and three disulphide-bonded intramolecular loops (Jones et al., 1999) (Fig.2).

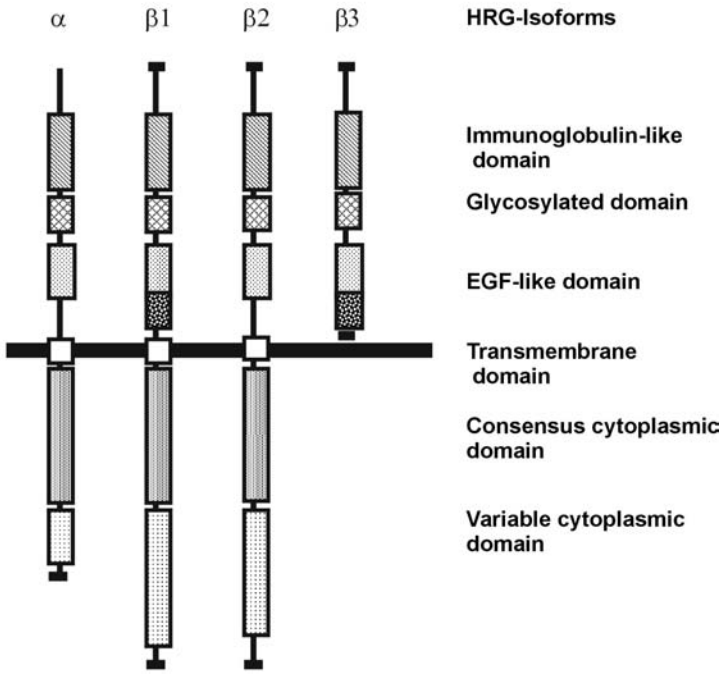

Fig.2: **Schematic representation of the human HRG family.**

This receptor-binding domain is usually part of a large transmembrane precursor containing other structural motifs such as immunoglobulin-like domain, heparin-binding sites and glycosylated linkers. Expression and processing of the precursor are highly regulated.

Neuregulins (NRGs) play a regulatory role in many cell systems (Buonanno and Fischbach, 2001). The effects exerted by NRGs on the glial cells during development has been extensively characterized (Garratt et al., 2000). NRGs are involved in the activation of astrocyte migration and have a particularly potent effect on their proliferation and differentiation by activating the MAPK pathway through SHC and PI_3-K (Faber-Elman et al., 1996; Wallasch et al., 1995). Four neuregulins, NRG1 - NRG4, are a subfamily of structurally related glycoproteins that are

produced by proteolytic processing of transmembrane precursors (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Fischbach and Rosen, 1997; Harari et al., 1999; Ishiguro et al., 1998; Zhang et al., 1997). The multitude of NRG1 isoforms, which include *neu* differentiation factor (NDF), neuronal acetylcholine receptor-inducing activity protein (ARIA), glial growth factor (GGF), sensorimotor-derived factor (SMDF) and the HRGs, reflects their multiple growth- and differentiation-regulating activities in a variety of different biological systems (Holmes et al., 1992). Expression of HRGs was detected in the central and peripheral nervous systems, where they exert biological activities at neuromusclar junctions, and junctions between neurons and Schwann cells, respectively (Orr-Urtreger et al., 1993). Other mebmers of the HRG family are important in the pathology of a wide variety of cell types and play a role in cancer progression (Lyne et al., 1997). It could be demonstrated that the most invasive breast cancer cell lines, lacking ER and consequently responsiveness to hormone therapy, overexpress HRG in an autocrine manner (deFazio et al., 2000).

2.3. The EGFR signalling network

2.3.1. General Overview

The components of the EGFR signalling pathway are evolutionarily ancient and, at first glance, resemble a simple growth factor signalling system: ligand binding to a monomeric receptor tyrosine kinase activates the cytoplasmic catalytic function by promoting receptor dimerization and self-phosphorylation on tyrosine residues (Yarden and Sliwkowski, 2001). The latter serve as docking sites for various adaptor proteins or enzymes, which simultaneously initiate many signalling cascades to produce a physiological outcome (Fig. 3). In higher eukaryotes, however, the simple linear pathway has evolved into a richly interactive, multilayered network, in which combinatorial expression and activation of components permits context-specific biological responses throughout development and adulthood.

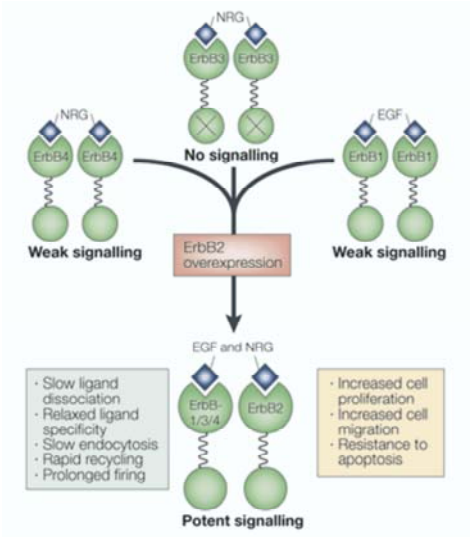

Fig. 3: **Signalling by ErbB homodimers in comparison with ErbB2/HER2-containing heterodimers.** Receptors are shown as two lobes connected by a transmembrane stretch. Binding of a ligand (EGF-like or NRG) to the extracellular lobe of ErbB1/EGFR, ErbB3/HER3 (note inactive kinase, marked by a cross) or ErbB4/HER4 induces homodimer formation. When ErbB2/HER2 is overexpressed, heterodimers form preferentially. Unlike homodimers, which are either inactive (ErbB3/HER3 homodimers) or signal only weakly, ErbB2/HER2-containing heterodimers have attributes that prolong and enhance downstream signalling (green box) and their outputs (yellow box). Apparently, homodimers of ErbB2/HER2 are weaker signalling complexes than heterodimers containing ErbB2/HER2. (taken from (Yarden and Sliwkowski, 2001)).

Examination of the intracellular and extracellular domains of the ErbBs provides a satisfactory explanation as to why a horizontal network of interactions is crucial to the ErbB signalling pathway: ErbB3/HER3 represents a RTK whose kinase activity is significantly weaker than that of the EGFR in a transphosphorylation reaction, which appears to be due to differences in two kinase domain amino acid residues that are conserved in other RTK family members (Kraus et al., 1989), whereas ErbB2/HER2 seems to have no direct ligand (Klapper et al., 1999). Therefore, in isolation neither ErbB2/HER2 nor ErbB3/HER3 can support linear signalling (Fig.3). Most inter-receptor interactions are mediated by ligands, and ErbB2/HER2-containing heterodimers are formed preferentially (Graus-Porta et al., 1997; Tzahar et al., 1996). Nevertheless, overexpression of a specific receptor can bias dimer formation, especially in the case of ErbB2/HER2, whose homodimers can spontaneously form in ErbB2/HER2-overexpressing cells. Many cancers of epithelial origin have an amplification of the ErbB2/HER2 gene, which pushes the equilibrium towards ErbB2/HER2 homodimer and heterodimer formation (Fig.3).

2.3.2. The Erb2/HER2-Erb3/HER3 signalling

The transmission of a mitogenic signal involves binding of HRG either to ErbB3/HER3 or to ErbB4/HER4, which then heterodimerize with ErbB2/HER2 and are thought to become transphosphorylated at their C-terminus by the ErbB2/HER2 kinase (Wallasch et al., 1995; Yarden and Sliwkowski, 2001). Upon HRG stimulation, ErbB2/HER2-ErbB3/HER3 heterodimers deliver the most potent and long lasting proliferative signal among the possible combinatorial pairs of HER members (Fig.4). This characteristic is due to the ability of ErbB2/HER2-ErbB3/HER3, but not other combinations, to continue signalling within the endosome after ligand-induced internalization (Lenferink et al., 1998; Yarden and Sliwkowski, 2001). Taken together, these data point out that not ErbB2/HER2 alone but the formation of ErbB2/HER2-ErbB3/HER3 complexes plays a crucial role in mitogenic signalling.

ErbB4, whose expression pattern is relatively limited, has several isoforms that differ in their juxtamembrane and carboxy-termini, resulting in differences in the recruitment of PI_3-K (Elenius et al., 1999), which activates cell-survival pathways. However, the role of ErbB4/HER4 and its signalling pathways in cancer are not clear.

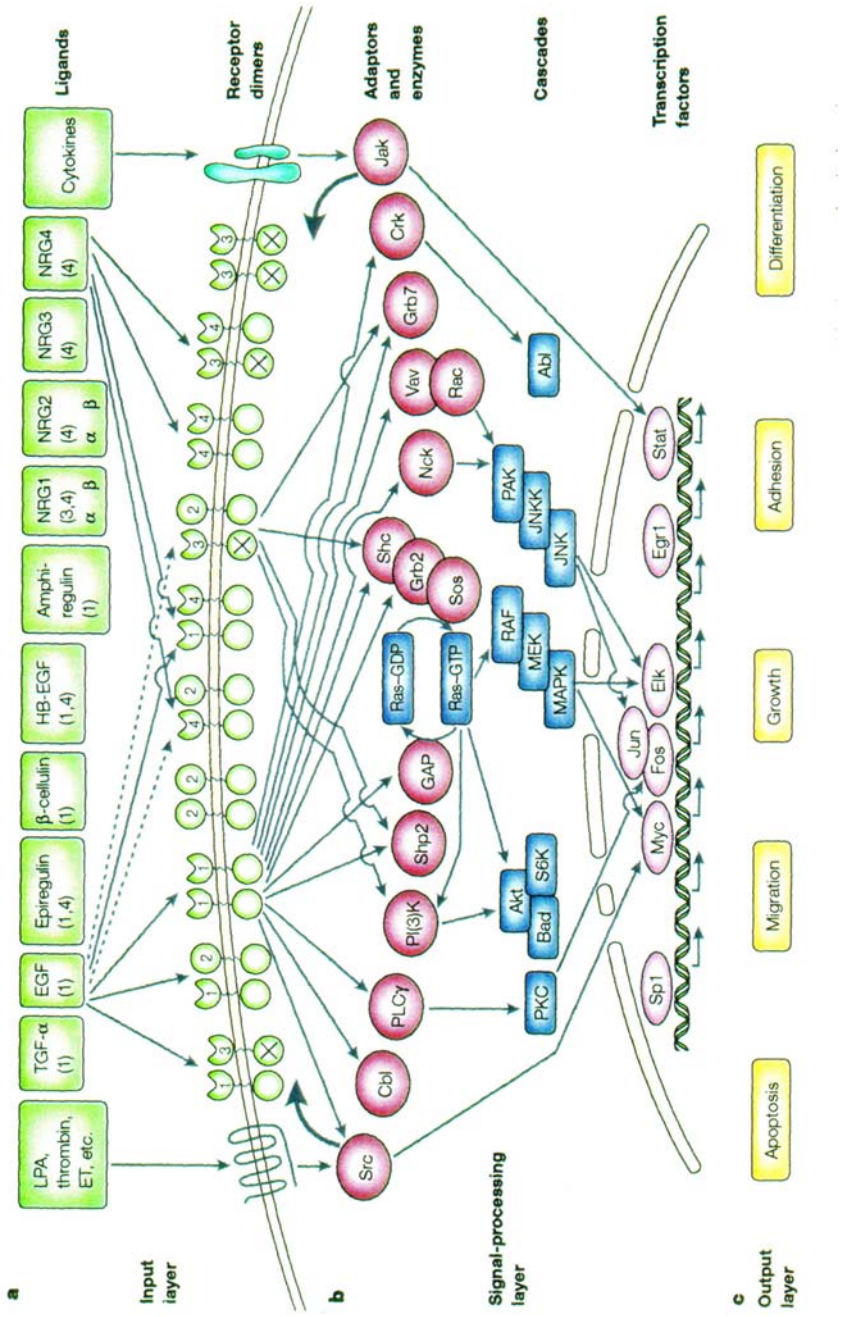

Fig. 4: **The ErbB signalling network.** (**a**) Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors (Jones et al., 1999). For simplicity, specificities of receptor binding are shown only for EGF and NRG4. ErbB2/HER2 binds no ligand with high affinity, and ErbB3/HER3 homodimers are catalytically inactive (crossed kinase domains). *Trans*-regulation by G-protein-coupled receptors (such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET)), and cytokine receptors is shown by wide arrows. (**b**) Signalling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1/EGFR homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. (**c)** How they are translated to specific types of output is poorly understood at present (taken from (Yarden and Sliwkowski, 2001)).

The Ras- and SHC-activated MAPK pathway is an invariable target of all ErbB ligands, and the PI_3-K-activated AKT pathway and p70S6K/p85S6K pathway are downstream of most active ErbB dimers. Signalling molecules, such as PI_3-K, SHC and GRB7, subsequently bind to the phosphorylated C-terminus of ErbB3/HER3 and thereby activate downstream pathways resulting in a mitogenic response (Fiddes et al., 1998). The potency and kinetics of PI_3-K activation differ, however, probably because PI_3-K couples directly with ErbB3/HER3 and ErbB4/HER4, but indirectly with ErbB1/EGFR and ErbB2/HER2 (Soltoff and Cantley, 1996). These findings indicate that ErbB3/HER3 is indeed a crucial coordinator of the ErbB signalling network.

However, while ErbB2/HER2 was the objective of intensive anti-cancer drug-designing studies, ErbB3/HER3, due to its reportedly impaired tyrosine kinase activity, was not considered by pharmacological research as a target so far.

2.4. PYK2-a member of the focal adhesion kinase (FAK) family

A recently identified member of the focal adhesion kinase family PYK2, also designated as FAK2, CAK-β, RAFTK or CADTK, was shown to be a link in G protein-coupled receptor-induced MAPK activation (Dikic et al., 1996; Girault et al., 1999; Lev et al., 1995). Phosphorylation of PYK2 leads to recruitment of Src-family kinases and to activation of extracellular signal-regulated kinases (ERKs). PYK2 is predominantly expressed in the central nervous system and in cells and tissues derived from the hematopoietic lineage, where it is found throughout the cytoplasm but concentrated in the perinuclear region (Andreev et al., 1999). PYK2 can be activated by stimuli that increase intracellular calcium levels (Avraham et al., 2000), and by stress factors such as hyperosmotic shock, UV light or tumour necrosis factor α, thereby inducing JNK (Tokiwa et al., 1996; Yu et al., 1996). However, the molecular details of the PYK2 activation mechanism are unknown.

2.5. Overview of cancer progression

It is generally accepted that malignant transformation involves genetic and epigenetic changes that derail common regulatory mechanisms and result in uncontrolled cellular proliferation and/or aberrant programmed cell death or apoptosis (Holliday, 1996; Simons, 1995). These cellular abnormalities, hallmarks of a carcinogenic process, are frequently associated with molecular alterations involving activation of proto-oncogenes and inactivation of tumour suppressor genes as a result of genetic predisposition and/or exposure to physical (e.g., radiation), chemical (e.g., carcinogens, dietary components) and biological (e.g., viruses) environmental factors (Couch, 1996). A central challenge for cancer biology is to understand the cellular and molecular processes that drive normal cells to neoplastic growth.

2.5.1. Gliomagenesis

Gliomas are a large collection of primary brain tumours that have morphology and gene-expression characteristics similar to glia, astrocytes and oligodendrocytes, which together support the functions of neurons in the brain (Berens and Giese, 1999). Because these tumours arise in the central nervous system and affect surrounding brain structures, patients with gliomas commonly develop symptoms that include headaches and seizures, or focal neurological alterations that cause weakness of one limb or language disturbance. Most gliomas arise sporadically and are not inherited within families; patients with gliomas however, frequently have a history of diverse cancer types. About 30,000 patients in the United States are newly diagnosed with a glioma each year (Greenlee et al., 2000).

Glioblastoma multiforme (GBM), the most malignant tumour of the primary central nervous system, arises from neoplastic transformation of glioblasts, which include type 1 and type 2 astrocytes. Additionally, GBMs are heterogenous intraparenchymal masses that show evidence of necrosis and hemorrhage (Holland, 2001). The central nervous system stem cell

gives rise to neuronal and glial progenitors, which subsequently give rise to the mature cell types found in the brain. Specific signal-transduction pathways have been shown to control the differentiation of precursor cells into mature glia. In cell culture, platelet-derived growth factor (PDGF) causes the oligodendrial progenitor population to proliferate (McKinnon et al., 1990), and cooperates with fibroblast growth factor 2 (FGF2) to prevent that population`s further differentiation into mature oligodendrocytes (Bogler et al., 1990; Mayer et al., 1993). Epidermal growth factor (EGF) and cilliary neurotrophic factor (CNTF) force glial progenitors towards astrocytic and oligodendrocytic differentiation (Fig.5) (Mayer et al., 1993; Rajan and McKay, 1998).

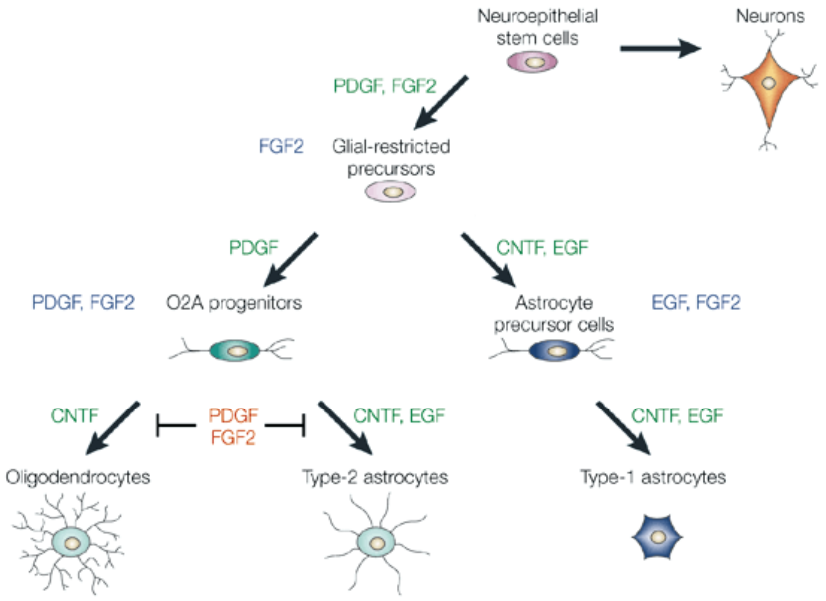

Fig.5: **Cellular differentiation in the central nervous system.** Stages of CNS cell differentiation; from stem cell to differentiated astrocyte, oligodendrocyte and neuron. Growth factors that promote the progression of one cell type to another are indicated in green, those that inhibit progression of one cell type to another are in red, and those that induce proliferation and maintain cells at a given stage of development are indicated in blue (taken from (Holland, 2001)).

During development, glioblasts migrate out of the subventricular zone of the brain into the developing white matter, while differentiating and proliferating en route (Altman, 1966; Goldman, 1992). This inherent ability of astrocytes to migrate represents a key feature of gliomas, during invasion of tumour cells into surrounding tissue. Although the effects of these growth factors on glial development are well documented, the understanding of how these pathways control cell fate and proliferation in glial development is still incomplete.

2.5.2. Breast cancer

Breast cancer is the most common malignancy among women in the US and accounts for one million new cases every year. It constitutes 30% of all new female cancers cases diagnosed, and accounts for 18% of female cancer deaths in the U.S. (Greenlee et al., 2000). Progress has been made in defining some of the critical processes associated with the development of breast cancer. However, the specific biochemical and molecular mechanisms underlying many of these complex carcinogenic events still remain to be elucidated.

The vast majority of breast cancers are carcinomas, the malignant tumours of the epithelia. Based upon histological evaluations, development of breast cancer has been postulated to be a multi-step process and follows a defined sequence of qualitatively different events (Russo et al., 1993; Russo et al., 1998) (Fig. 6), as documented for a number of other malignancies (Farber, 1984; Klein and Klein, 1985). In human breast, ductal hyperplasia and atypical ductal hyperplasia represent the initial stages of neoplastic growth and progress gradually to ductal carcinoma *in situ*, invasive ductal carcinoma and ultimately metastasis, even though normal cells could directly give rise to ductal carcinoma *in situ* or invasive ductal carcinoma (Page and Dupont, 1990; Russo et al., 1993) (Fig. 6).

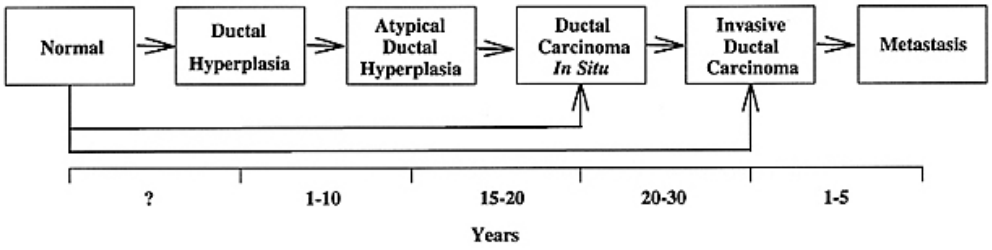

Fig. 6: ***In vivo* model of neoplastic progression of human breast carcinomas.**

Growth factors that bind to and activate receptor tyrosine kinases expressed in epithelial cells have distinct roles in mammary gland development. Interestingly, overexpression of many of these ligands has also been associated with mammary tumourigenesis. Several groups have established transgenic mice bearing TGF-α, an EGFR-specific ligand, under the control of either the MMTV-LTR (mouse mammary tumour virus-long terminal repeat) or metallothionein promoter (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990).

In two studies, TGF-α expression resulted in epithelial hyperplasia and dysplasia, with only a small number of multiparous animals developing mammary tumours (Matsui et al., 1990; Sandgren et al., 1990).

The remaining study reported reduced penetration of the mammary ducts into the fat pad and an increase in the number of actively dividing epithelial cells, which were not restricted to the terminal end buds (Jhappan et al., 1990). More recently, a member from the NDF (*Neu* differetiation factor) family of ligands, which bind directly to ErbB3/HER3 and ErbB4/HER4, has also been found to be expressed in the mammary glands of transgenic mice (Krane and Leder, 1996).

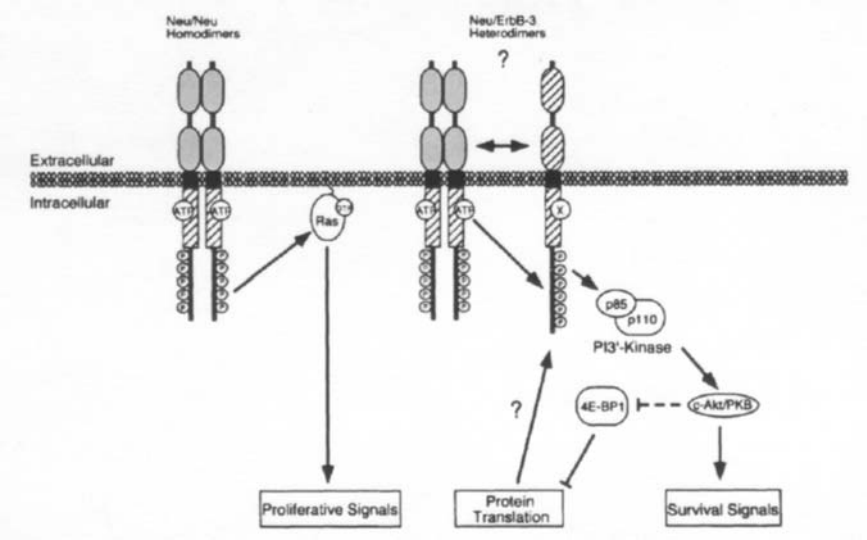

Fig. 7: **Hypothetical model for the role of ErbB3/HER3 in *Neu*-induced mammary tumourigenesis.** ErbB2/HER2 homodimers are formed as a consequence of somatic mutations or ligand-induced activation, leading to activation of Ras-GTP and induction of cell proliferation. Activation of *Neu* results in the transphosphorylation of ErbB3/HER3, which, in turn, recruits the PI_3-K pathway. Activation of PI_3-K stimulates AKT/PKB, which then provides a cell survival signal. A potential mechanism by which translation of ErbB3/HER3 may be upregulated via inhibition of 4E-BP1 is also shown (taken from (Siegel et al., 2000)).

Multiparous animals expressing NDF-β2 develop palpable mammary tumours at an average age of 357 days. These data demonstrate that ligands that bind and activate different members of the EGFR family exert similar proliferative effects on mammary epithelial cells (Fig. 7). This may reflect the ability of TGF-α and NDF-β2 to induce heterodimers between various EGFR family members, with *Neu* representing a common component of the activated receptor complex. Finally, it is evident that both ligands are necessary, but not sufficient, for mammary tumourigenesis given the long latency and focal nature of the tumours that arise in these mice.

2.6. Therapeutic antibodies in cancer treatment

2.6.1. General overview

Antibodies have for many decades been anticipated as the ideal molecule for the therapy of cancer and infectious diseases (Borrebaeck, 1989). This particular therapeutic modality has grown to the point where it constitutes approximately 30% of all clinical trials (excluding vaccine and gene therapy) registered by the Food and Drug Administration (FDA) in the USA (Borrebaeck, 1999) (Tab. 1).

Product name	Specificity	Indication	Year of approval
Orthoclone OKT®3 (muromonab-CD3)	CD3	Acute kidney transplant rejection	1986
ReoPro® (abciximab)	gpIIb/IIIa	Prevention of bloodclotting Refractory unstable angina	1994
Rituxan® (rituximab)	CD20	Non-Hodgkin`s lymphoma	1997
Zenapax® (daclizumab)	CD25 (IL2R)	Acute kidney transplant rejection	1997
Remicade® (infliximab)	TNF-α	Crohn`s disease Rheumatoid arthritis	1999
Simulect® (basiliximab)	CD25 (IL2R)	Acute kidney transplant rejection	1998
Synagis® (palivizumab)	RSV	Respiratory syncytia virus	1998
Thymoglobulin®	Polyclonal Abs	Acute kidney transplant rejection	1998
Herceptin® (trastuzumab)	HER2	Metastatic breast cancer	1998
Nabi-HB™	Polyclonal Abs	Hepatitis B	1999
CroFab®	Snake venom	Rattlesnake antidote	2000
Campath® (alemtuzumab)	CD52	Chronic lymphocytic leukaemia	2001
Mylotarg™ (gemtuzumab, ozogamicin)	CD33	Acute myeloid leukaemia	2000

Tab. 1: **FDA-approved therapeutic antibodies.**[1]

[1] New Medicines in Development: Biotechnology: a 2000 survey at URL:http://www.pharma.org/searchcures/newsmeds/biotech2000/bio00.pdf

Despite the fact that work on antibodies targeting intracellular molecules is being performed, most targets for therapeutic antibodies are extracellular molecules (Marasco and Dana Jones, 1998). Depending on the indication, the type of targets may differ significantly as may the need for partial or total antibody-mediated effect. Targeting tumour cells, a total (or close to total) elimination of all malignant cells is fundamental, putting tremendous demands on the distribution of the target molecule, the ability of the therapeutic antibody to reach and bind to all tumour cells and the effector system mediating the toxic effect to the tumour cell. In contrast, antibodies that exert their function through blocking signalling between or interfering with, for example, TNF–TNFR (tumour necrosis factor receptor) or IL-2–IL-2R (interleukin-2 receptor) interactions may be effective even if the blocking is not 100% efficient (Feldmann and Maini, 2001) (Berard et al., 1999).

Many therapeutic antibodies have been designed to exert their effect when coupled to immunotoxins, drugs or radioisotopes (Goldenberg, 1999; Riethmuller et al., 1998). The effector mechanisms associated with naked antibodies have been considered to be associated with the ability of the antibody to recruit natural effector mechanisms like ADCC (antibody-dependent cellular cytotoxicity) and CDC (complement-dependent cytotoxicity). Target molecules that are normally involved in cell signalling-for example, those that control cell growth or are involved in mechanisms participating in apoptosis-might be ideal targets for naked therapeutic antibodies. Interestingly, cells normally considered to mediate ADCC might also aid cytotoxic activity through the cross-linking of the therapeutic antibody on the surface of the target cell via their Fcγ-receptors, in addition to the ADCC effect (Cragg et al., 1999; Shan et al., 2000). This suggests that novel targets for cancer therapy should be sought among functionally associated surface molecules.

2.6.2. Herceptin®-the "gold standard" in antibody therapy

The development of a monoclonal antibody against ErbB2/HER2, 4D5, and its subsequent humanized counterpart Herceptin® (Trastuzumab), which has recently been approved by the FDA, represents the hitherto state-of-the-art agent against ErbB2/HER2-overexpressing metastatic breast carcinoma (Fendly et al., 1987; Hudziak et al., 1989; Stebbing et al., 2000). Therapeutically effective antibodies, like Herceptin® or Rituxan®, have been shown to act through two different mechanisms, i.e. the engagement of the effector cells of the immune system as well as a direct cytotoxic, apoptosis inducing, effect (Baselga and Albanell, 2001; Sliwkowski et al., 1999; Soderlind et al., 2000; Vitetta and Uhr, 1994). Multinational studies have employed Herceptin® in protocols of combined therapy, prolonging significantly the life span of the patients (Cobleigh et al., 1999; Shak, 1999; Slamon et al., 2001).

3. Objectives

Members of the EGFR family of receptor tyrosine kinases have been frequently implicated as causal agents in manifestation and progression of human cancer. Currently, the ErbB1/EGFR and ErbB2/HER2 are successfully pursued as targets for anti-tumour therapy. In particular, ErbB2/HER2 has been under rigorous investigation and the therapeutical antibody Herceptin® has emerged and has subsequently been approved for the treatment of metastatic breast cancer.

In this thesis, we address the functional role and signalling mechanisms of the third member of the EGFR-family, HER3 in gliomas. Additionally, we also set out to clarify the role of PYK2 tyrosine phosphorylation in human glioma cell lines upon Heregulin stimulation. Finally, we explore the inhibitory potential of a monoclonal antibody against ErbB3/HER3 (α-HER3^{ECD}) on signal transduction processes in breast cancer cells and compare its effector functions with those of Herceptin®.

4. Methods

4.1. Materials and general methods

Media were purchased from Gibco, fetal bovine serum (FBS), horse serum and Collagen Type IV were from Sigma. Hybond ECL membranes and γ-^{32}P-ATP were purchased from Amersham, PP1, AG825, Wortmannin (WT), PD98059 (Alessi et al., 1995b) and Ionomycin (IONO) from Calbiochem and Herceptin® (HC) from Roche. Antibodies raised against following proteins were used: PYK2 (polyclonal goat antibody N19, Santa Cruz, and polyclonal rabbit antibody (pAb) Upstate Biotechnology, Inc. (UBI)), ERK-2 (pAb C14, K23, Signal Transduction), SHC (pAb (Seedorf et al., 1994), mAb, Affiniti), ErbB2/HER2 (monoclonal mouse antibody (mAb) 2-13D), ErbB3/HER3 (mAb 2F12 ($IgG_{2a\kappa}$), UBI, mAb (IgG_1) (α–$HER3^{ECD}$), UBI), p85 (mAb UB93-3, UBI), GRB2 (mAb 2F12, UBI), VSV (mAb P5D4, Boehringer Mannheim), and phosphotyrosine (mAb 4G10, UBI). HRP-coupled secondary antibodies were purchased from Biorad, flourochrome-coupled secondary antibodies from Molecular Probes. Transwell chambers (0.3 cm^2, 8 μm) were purchased from Costar. Growth Factor Reduced Matrigel (GFRM) was purchased from Collaborative Biomedical Products. Thin-layer Chromatography plates (Silica Gel 60) precoated with oxalate were from Merck. Cell lines HEK293, rat C6 and PC12 and human SF763, SF767, PhoenixA and MDA-MB-468 were obtained from ATCC and cultured according to the supplier`s protocol. The human cell line MCF-7^{ADR} was obtained from DKFZ (Heidelberg) and cultured according to the suppliers' protocols. Tetracyclin-inducible PC12 system stably expressing PYK2-KM (Tet-off) was described previously (Zwick et al., 1999).

4.2. Purification of recombinant proteins

Recombinant human GST-α-HRG fusion protein (α-HRG), GST-PYK2-CT, GST-GRB2 and GST-c-Jun were produced in E. coli and purified as described (Wallasch et al., 1995) or according to the purification protocol for pGEX fusion proteins (Pharmacia). GST-HER2-KD was generated by using the pRK5 construct and amplifying the kinase domain of HER2 (a.a. 726-1012 by PCR. GST-HER3-KD was generated by using the pcDNA3.1-HER3 construct and amplifying the kinase domain of HER3 (a.a. 730-1065) by PCR.The purified recombinant proteins were dialyzed overnight against PBS and 10% glycerol, aliquoted and stored at –80°C.

4.3. Immunoflourescence studies and confocal microscopy

Briefly, SF763 and SF767 ($3x10^5$ cells) were grown on coverslips and starved for 24 h. After stimulation with 5 μg ml^{-1} HRG for 20 min, cells were fixed with 3.7% formaldehyd and permeabilized with 0.2% saponin (Sigma) in 3% BSA (Sigma). Blocking was performed with 3% BSA for 1 h. PYK2 and ErbB3/HER3 proteins were labeled with the indicated primary antibodies and stained using a fluorochrome-coupled donkey anti-goat α-488 secondary antibody for PYK2, and TRITC-coupled rabbit anti-mouse secondary antibody for ErbB3/HER3 (Molecular Probes). Confocal images were taken with an LSM 410 microscope using a 40¥/1.3 Neofluar objective (Zeiss). For the GFP fluorescence, the 488 nm argon-ion laser band was used for excitation together with a 510-525 nm band-pass emission filter; for TRITC, the 543 nm helium-neon-ion band and a 570 nm high-pass filter; and for DAPI, the 364 nm argon-ion band and a 400-435 nm band-pass filter (Weber et al., 1999).

4.4. Plasmid constructs and site-directed mutagenesis

pcDNA3.1-PYK2-VSV and pcDNA3.1-PYK2-KM-VSV constructs were generated using the pRK5 constructs and standard methods. PYK2-KM was generated as described (Zwick et al., 1999). GST-PYK2-CT was generated by using the pRK5 construct and amplifying the C-terminus of PYK2 by PCR (positions 716-1009). The fragment was subcloned into the procaryotic expression vector pGEX-5X1 (Pharmacia). Tyrosine to phenylalanine mutations in ErbB3/HER3 were performed using the pcDNA3.1-ErbB3/HER3 construct and the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturers protocol. Correct incorporation of the mutations was verified by DNA sequencing.

4.5. Transient overexpression of PYK2, PYK2-KM, ErbB2/HER2, ErbB2/HER2-KM, ErbB3/HER3, and ErbB3/HER3-KM proteins in eukaryotic cells

The HEK293 cell system was used for transient protein expression. HEK293 cells were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin (100 IU ml^{-1}) at 7.5% CO_2 and 37˚C. Transfections were carried out using a modified calcium phosphate method (Chen and Okayama, 1987). Briefly, $3.5x10^5$ cells were incubated overnight in 3 ml of growth medium. 1 µg of supercoiled DNA was mixed with 0.25 M $CaCl_2$ solution in a final volume of 400 µl. The mixture was added to the same volume of 2x transfection buffer (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) and incubated for 15 min at room temperature before it was added dropwise to the cells. After incubation for 12 h at 37˚C under 3% CO_2, the medium was removed, cells were washed twice with PBS and were then starved for 24 h in DMEM supplemented with 0.1% FCS.

4.6. Western Immunoblotting I

SF763, SF767 or transfected HEK293 cells were either left untreated or were pretreated with PP1 (10 μM), AG825 (10 μM), Wortmannin (WT) (100 nM) and PD98059 (25 μM) for 30-60 min following stimulation with 5 μg ml^{-1} recombinant human α-HRG for 20 min or with 5 μM IONO for 5 min at 37°C. Upon α-HRG or IONO stimulation, the cells were lysed on ice in a lysis buffer (50 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM Sodiumflouride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 10 mg ml^{-1} aprotinin). Crude lysates were centrifuged at 12500 g for 20 min at 4°C. For immunoprecipitations, the appropriate antiserum and 30 μl of protein A-Sepharose (Pharmacia) was added to the cleared lysate and incubated for 3 h at 4°C. Immunoprecipitates were washed with a washing buffer (20 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM Sodiumflouride 10% (v/v) glycerol, 1% (v/v) Triton X-100). Sample buffer containing SDS and 2-mercaptoethanol was added and the samples were denaturated by heating at 95°C for 4 min.

Proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, nitrocellulose filters were first incubated with mouse monoclonal or rabbit polyclonal primary antibodies for 3 h at 4°C. Next, a HRP-coupled goat anti-mouse or goat anti-rabbit secondary antibody was added (Biorad), followed by an enhanced chemoluminescence (ECL) substrate reaction (Amersham). The substrate reaction was detected on Kodak X-Omat film. Filters that were used more than once with different antibodies were stripped according to the manufacturer's protocol, blocked and reprobed.

4.7. Western Immunoblotting II

MCF-7^{ADR} and MDA-MB-468 cells were either left untreated or were pretreated with Herceptin (HC) (10 μg/ml), α-HER3^{ECD} (10 μg/ml) and Wortmannin (WT) (100 nM) for 30-60 min following stimulation with 5 μg ml-1 recombinant human HRG for 5 min at 37˚C. Upon HRG stimulation, the cells were lysed on ice in a lysis buffer (50 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM Sodiumflouride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 10 mg ml^{-1} aprotinin). Crude lysates were centrifuged at 12500 g for 20 min at 4˚C. For immunoprecipitations, the appropriate antibody and 30 μl of protein A-Sepharose (Pharmacia) were added to the cleared lysate and incubated for 3 h at 4˚C. Immunoprecipitates were washed with a washing buffer (20 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM Sodiumflouride 10% (v/v) glycerol, 1% (v/v) Triton X-100). Sample buffer containing SDS and 2-mercaptoethanol was added and the samples were denaturated by heating at 95˚C for 4 min.

Proteins were fractionated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, nitrocellulose filters were first incubated with mouse monoclonal or rabbit polyclonal primary antibodies for 3 h at 4˚C. Next, a HRP-coupled goat anti-mouse or goat anti-rabbit secondary antibody was added, followed by an enhanced chemiluminescence (ECL) substrate reaction (Amersham). The substrate reaction was detected on Kodak X-Omat film. Filters that were used more than once with different antibodies were stripped according to the manufacturer's protocol, blocked and reprobed.

4.8. Generation of recombinant retroviruses and retrovirus-mediated gene transfer

Briefly, pLXSN-PYK2 and pLXSN-PYK2-KM were generated by cloning an EcoRI-XhoI fragment from pRK5 carrying the cDNAs of PYK2-WT and kinase-inactive PYK2, K457M (PYK2-KM), respectively, into pLXSN. Amphotrophic virus titer, which was generated by transient transfection of retrovirus expression plasmids into the virus producer cell line PhoenixA (ATCC), was determined by infecting NIH-3T3 cells with serial dilutions of retrovirus-containing, cell-free PhoenixA supernatants and counting the number of G418-resistant colonies. The titers were approximately $1x10^6$ cfu ml^{-1} both for PYK2 and PYK2-KM virus supernatants. Subconfluent C6, SF763 and SF767 cells ($9x10^5$ cells) were incubated with supernatants of cells releasing high titers of pLXSN-PYK2 or pLXSN-PYK2-KM viruses ($1x10^6$ G418 cfu ml^{-1}) for 24 h in the presence of Polybrene (4 mg ml^{-1}, Aldrich).

4.9. Cell surface biotinylation

Monolayers of cells grown in 10-cm dishes were washed three times with ice-cold phosphate-buffered saline and then incubated with 0.5 mg/ml of a water-soluble Biotin-X-NHS (Calbiochem) dissolved in borate buffer (10 mM boric acid, 150 mM NaCl, pH 8.0) for 45 min at 4 °C. Coupling of biotin was blocked by extensive washes with a solution of 15 mM glycine in phosphate-buffered saline. Cells were then either treated with HRG (5 µg/ml) or with α-$HER3^{ECD}$ antibody for different time intervals at 37 °C. To evaluate the amounts of cell surface receptors, the cells were subjected to immunoprecipitation and gel electrophoresis. Visualization of the biotinylated proteins was performed by probing the nitrocellulose membranes with horseradish peroxidase-conjugated streptavidin (Amersham Pharmacia Biotech) and developed with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

4.10. *In vitro*-Kinase Assays

4.10.1. ErbB3/HER3-Kinase Assay

HER3 kinase assays were performed using either HER2 or HER3 immunoprecipitates or 500 ng recombinant GST-HER2-KD GST-HER3-KD. Cultured cells were lysed on ice in a lysis buffer (50 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM Sodiumflouride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 10 mg ml^{-1} aprotinin). Crude lysates were centrifuged at 12500 g for 20 min at 4˚C. Endogenous ErbB3/HER3 is immunoprecipitated from the supernatants (2 mg of protein) by incubation for 3.5 h with primary ErbB3/HER3 antibody (mAb 2F12, UBI) prebound to protein A beads. The pellets are washed twice with lysis buffer and thrice with a kinase buffer composed of 25mM HEPES (pH 7.5), 20 mM β–glycerophosphate, 7.5 mM $MgCl_2$, 7.5 mM $MnCl_2$, 1 mM DTT, and 0.1 mM sodium orthovanadate. Before kinase reaction was started immunoprecipitates or GST-fusions were equiliberated by adding 30 µl kinase reaction buffer including 10 µg GST-PYK2-CT or MBP for 2 minutes at 30 °C. Kinase reaction was started by adding 10 µM ATP (including 10 µCi γ-^{32}P-ATP), incubated for 30 minutes at 30°C and by adding 30 µl Lämmli-buffer. The kinase reactions are initiated by addition of 10 µCi of [γ-^{32}P]-ATP, 15 µM “cold” ATP, and 5 µg of GST-PYK2-CT. The reaction mixture in a final volume of 40 µl is incubated at 30°C for 30 min and the reaction is terminated by addition of sample buffer containing SDS and 2-mercaptoethanol and the samples were denaturated by heating at 95˚C for 4 min. The reaction products are separated by electrophoresis on SDS-10% (w/v) polyacrylamide gels, and the gels either stained with Coomassie (G250) or blotted on a nylon membrane and the ^{32}P-labeled proteins visualized by autoradiography. To determine equal protein loading, the membrane is stripped and reprobed with an specific anti-ErbB3/HER3 antibody.

4.10.2. MAPK Assay

Cultured cells were lysed on ice in a lysis buffer (50 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM Sodiumflouride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 10 mg ml^{-1} aprotinin). Crude lysates were centrifuged at 12500 g for 20 min at 4˚C. Endogenous ERK-2 is immunoprecipitated from the supernatants (150 µg of protein) by incubation for 1.5 h with primary ERK-2 antibody (pAb K-23, SC) prebound to protein A beads. The pellets are washed twice with lysis buffer and twice with a kinase buffer composed of 25mM HEPES (pH 7.5), 20 mM β–glycerophosphate, 20 mM PNPP, 20 mM $MgCl_2$, 2 mM DTT, and 0.1 mM sodium orthovanadate. The kinase reactions are initiated by addition of 1 µCi of [γ-^{32}P]-ATP, 15 µM "cold" ATP, and 2 µg of myelin basic protein (MBP). The reaction mixture in a final volume of 40 µl is incubated at 30°C for 20 min and the reaction is terminated by addition of sample buffer containing SDS and 2-mercaptoethanol and the samples were denaturated by heating at 95˚C for 4 min. The reaction products are separated by electrophoresis on SDS-12.5% (w/v) polyacrylamide gels, blotted on a nylon membrane and the ^{32}P-labeled proteins are visualized by autoradiography. To determine equal protein loading, the membrane is stripped and reprobed with an specific anti-ERK-2 antibody.

4.10.3. JNK Assay

Cultured cells are lysed in lysis buffer containing 20 mM Tris (ph 7.6), 0.5% (v/v) Nonidet P-40 (NP-40), 250 mM NaCl, 3 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenymethylsulfonyl fluoride (PMSF), 20 mM β–glycerophosphate, 1 mm sodium orthovanadate, and leupeptin (1µg/ml). The lysates are cleared by centrifugation at 15,000g for 15 min at 4°C. Endogenous JNK1 is immunoprecipitated from the supernatants (300 µg of protein) by incubation for 3 h with primary antibody JNK-1 (pAB, C-17 SC) prebound to protein A beads. The pellets are washed twice with lysis

buffer and twice with a kinase buffer composed of 25mM HEPES (pH 7.5), 20 mM –glycerophosphate, 20 mM PNPP, 20 mM $MgCl_2$, 2 mM DTT, and 0.1 mM sodium orthovanadate. The kinase reactions are initiated by addition of 5 µCi of [γ-^{32}P]-ATP, 20 µM "cold" ATP, and 1 µg of glutathione S-transferase (GST)-c-jun (a.a. 1-79). The reaction mixture in a final volume of 25 µl is incubated at 30°C for 30 min and the reaction is terminated by addition of sodium docecyl sulfate (SDS) sample buffer and boiled for 5 min in tightly capped tubes. The reaction products are separated by electrophoresis on SDS-12.5% (w/v) polyacrylamide gels, blotted on a nylon membrane and the ^{32}P-labeled proteins are visualized by autoradiography. To determine equal protein loading, the membrane is stripped and reprobed with an specific anti-JNK-1 antibody (Sudo and Karin, 2000).

4.10.4. PI_3-K Assay

Briefly, tyrosine phosphorylated proteins were precipitated from HRG-treated or untreated cells as described above. The immunoprecipitates were washed four times with 1 ml of the following buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 10% (v/v) glycerol, 0.2 mM sodium orthovanadate. After the last wash 40 µl aliquots of a master mix consisting of 10 µl 5X buffer (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 25 mM $Mg(CH_3COO)_2$, 25 mM $MnCl_2$, 2.5 mM EGTA), 10 µl phosphatidylinositol + phosphatidylserine (0.5 mg/ml each in 20 mM HEPES, pH 7.5), 5 µl 0.2 mM ATP, 5 µl [γ-^{32}P]-(10 µCi per sample) ATP solution and 5 µl H_2O were added to the immunoprecipitates and the kinase reaction was stopped after incubation at 30°C for 30 min by the addition of 100 µl of 1 M HCl and 100 µl of methanol-chloroform [1:1 (v/v) mixture]. The lipid-containing organic phase was resolved on oxalate-coated, thin-layer chromatography plates (Silica Gel 60; Merck) developed in chloroform:methanol:water:glacial acetic acid:acetone (40:13:7:12:15 v/v). The plates were then dried and exposed for autoradiography and/or phosphoimager (Fuji) analysis (Alessi et al., 1995a; Morgan et al., 1990).

4.11. Proliferation Assay

Proliferation was measured performing a BrdU-Incorporation assay (Roche) according to the manufacturers` protocol.

4.12. Chemotaxis Assays

4.12.1. Migration Assay

Migration assay (Chemotaxis) was performed as follows: briefly, $2x10^5$ cells were plated on transwell chambers precoated with 4 µg Collagen Type IV. Conditioned NIH-3T3 medium was used as a chemoattractant (Albini et al., 1987). Following 16 h of incubation, non-migrating cells were removed with cotton swabs, whereas invading cells were fixed, stained with Crystal violet and counted under bright-field illumination using an Axiovert135 inverted microscope (Zeiss). Counts from 4 filters for each strain were pooled and compared among different strains using ANOVA.

4.12.2. Tumor invasion Assay

Tumor invasion assay was performed as described previously (Albini et al., 1987). Briefly, $3x10^5$ cells were plated on transwell chambers precoated with 100 µg GFRM. Conditioned NIH-3T3 medium was used as a chemoattractant. Cells were stimulated with 5 $\mu g ml^{-1}$ α-HRG during the experiment. Following 16 h of incubation, non-invading cells were removed with cotton swabs, whereas invading cells were fixed, stained with Crystal violet and counted under bright-field illumination using an Axiovert135 inverted microscope (Zeiss). Counts from 4 filters for each strain were pooled and compared among different strains using the two-tailed t-test.

5. Results

5.1. Tyrosine-phosphorylation of PYK2 is dependent on ErbB2/HER2 and ErbB3/HER3

PYK2 gets tyrosine-phosphorylated in the human glioma cell line SF767 upon stimulation by α-HRG (Fig. 8a). In order to evaluate the mechanism of HRG-induced PYK2 tyrosine-phosphorylation, we inhibited two candidate protein tyrosine kinases, src and ErbB2/HER2, since it had been reported that src kinase associates with ErbB2/HER2 after HRG-stimulation and phosphorylates PYK2 upon GPCR stimulation (Dikic et al., 1996). Treatment of cells with the src inhibitor PP1 prior to HRG stimulation had no effect on PYK2 phosphorylation on tyrosines indicating that src does not mediate PYK2 tyrosine-phosphorylation after HRG treatment. In contrast, stimulation of SF767 cells by Ionomycin, which leads to an influx of Ca^{2+}-ions as in the case of GPCR stimulation (Zwick et al., 1999), induces tyrosine-phosphorylation of PYK2 that is dependent on src activity (Fig. 8a, left vs. right panel, lanes 3 and 4).

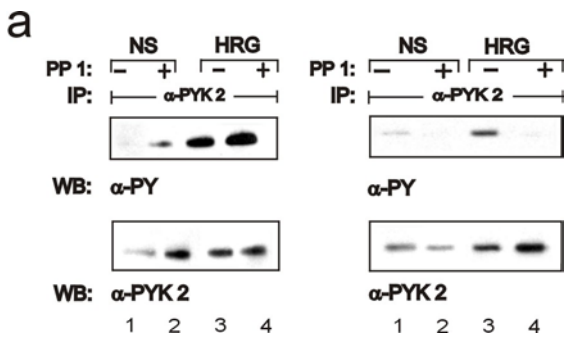

Fig. 8a: **Effects of a c-src inhibitor PP1 on PYK2 tyrosine phosphorylation.** Tyrosine phosphorylation of PYK2 is independent of c-src upon α-HRG stimulation, in contrast to IONO stimulation. SF767 gliomas were pretreated with 5 μM PP1 for 30 minutes and stimulated either with 5 μg ml^{-1} Heregulin (α-HRG, left panel) or 5 μM Ionomycin (IONO, right panel) for 20 min and 5 min, respectively. Tyrosine phosphorylation level was analysed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-PY)

(**a**, upper panel). Equal loading of proteins was checked by reblotting with α-PYK2 (**a**, lower panel). Unstimulated cells are indicated by NS.

It has been reported that in the breast carcinoma cell line MDA-MB-435 HRG-induced activation of ErbB2/HER2, which is mediated by heterodimerization between ErbB2/HER2 and ErbB3/HER3, leads to tyrosine-phosphorylation of PYK2 (Zrihan-Licht et al., 2000). As shown in figure 8b, in SF767 glioma cells a tyrosine phosphorylated protein of M_r=113 kDa, which coprecipitates with ErbB3/HER3 prior to stimulation with HRG was identified as PYK2 (Fig. 8b, upper and middle panels, lanes 5-8). In contrast, under the same conditions precipitation of ErbB2/HER2 reveals no association with PYK2 (Fig. 8b, upper and lower panel, lanes 1-4). Upon HRG-stimulation tyrosine-phosphorylation of PYK2 increases (Fig. 8b, upper panel, lanes 5 and 7), but is attenuated in the presence of the ErbB2/HER2 inhibitor AG825 (Fig. 8b, upper panel, lanes 6 and 8), indicating that tyrosine-phosphorylation of PYK2 is dependent on ErbB2/HER2 kinase activity. The amount of PYK2 that coprecipitates with ErbB3/HER3 is not elevated by HRG-stimulation (Fig. 8b, middle panel, lanes 5 and 7), but decreases after addition of AG825 (Fig. 8b, middle panel, lanes 6 and 8). Tyrosine-phosphorylation of PYK2 was, however, attenuated in the presence of the ErbB2/HER2 inhibitor AG825 (Fig. 8b, lanes 9-12). Precipitation of PYK2 under the same conditions showed a weak but reproducibly specific association with ErbB3/HER3 (Fig. 8b, lanes 9-12), in a clear contrast to ErbB2/HER2, which showed no coprecipitation. These results suggest a constitutive association of PYK2 with ErbB3/HER3, which is dependent on ErbB2/HER2 kinase activity. The same results were also obtained in the glioma cell line SF763 (data not shown), and suggest a constitutive association of PYK2 with ErbB3/HER3, which is ErbB2/HER2 kinase dependent.

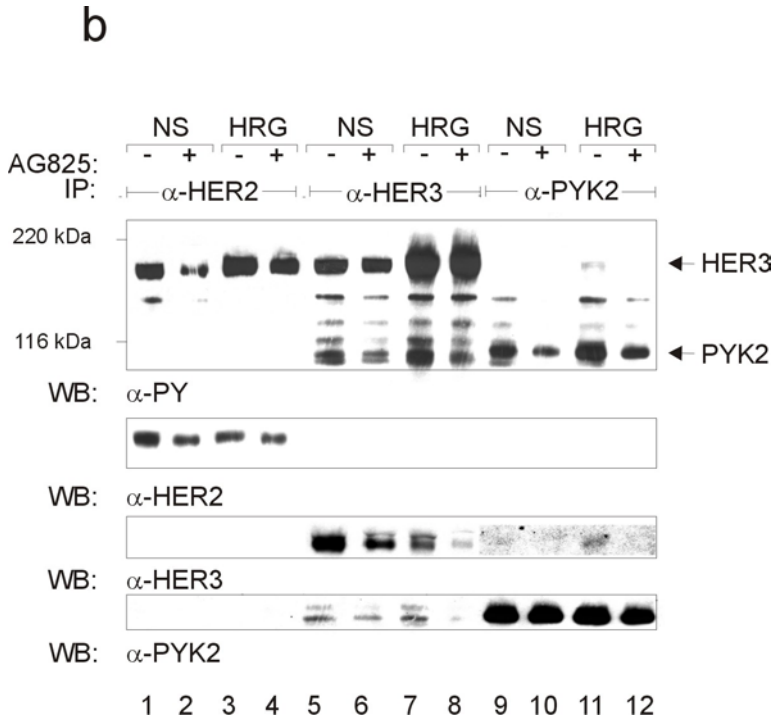

Fig. 8b: **Effects of a ErbB2/HER2 inhibitor AG825 on PYK2 tyrosine phosphorylation.** PYK2 coprecipitation with ErbB3/HER3 depends on the ErbB2/HER2 kinase activity, and tyrosine phosphorylation of PYK2 is proportional to its binding to ErbB3/HER3. SF767 gliomas were pretreated with 10 μM AG825 for 1 hour and stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG). Cell lysates were subjected to immunoprecipitation (IP) using polyclonal anti-PYK2 (α-PYK2) or monoclonal anti-ErbB3/HER3 (α-ErbB3/HER3) antibodies. Tyrosine phosphorylation level was analysed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-PY) (**b**, upper panel). Equal loading of proteins was checked by reblotting with α-PYK2, α-ErbB3/HER3 and α-ErbB2/HER2 antibodies, respectively (**b**, upper middle, middle and lower panels). PYK2 coprecipitating with ErbB3/HER3 was detected by probing the membrane with α-PYK2 antibody (**b**, lower panel, lanes 5-8). ErbB3/HER3 coprecipitating with PYK2 was detected by probing the membrane with α-ErbB3/HER3 antibody (**b**, middle panel, lanes 9-12). Unstimulated cells are indicated by NS.

To provide further support for the PYK2/ErbB3/HER3 interaction, we performed immunoflourescense studies in SF763 and SF767 cell lines using a laser scanning confocal microscope (Fig. 9). As shown in figure 9, in both unstimulated and HRG-stimulated SF763 and SF767 cells, PYK2 is predominantly localized to the perinuclear cytoplasm in a punctuated pattern, which is predominantly coincident with that of ErbB3/HER3 as demonstrated by image overlay. Thus, biochemical as well as cell biological immunofluorescense analysis supports the conclusion that PYK2 and ErbB3/HER3 are constitutively associated in glioma cells.

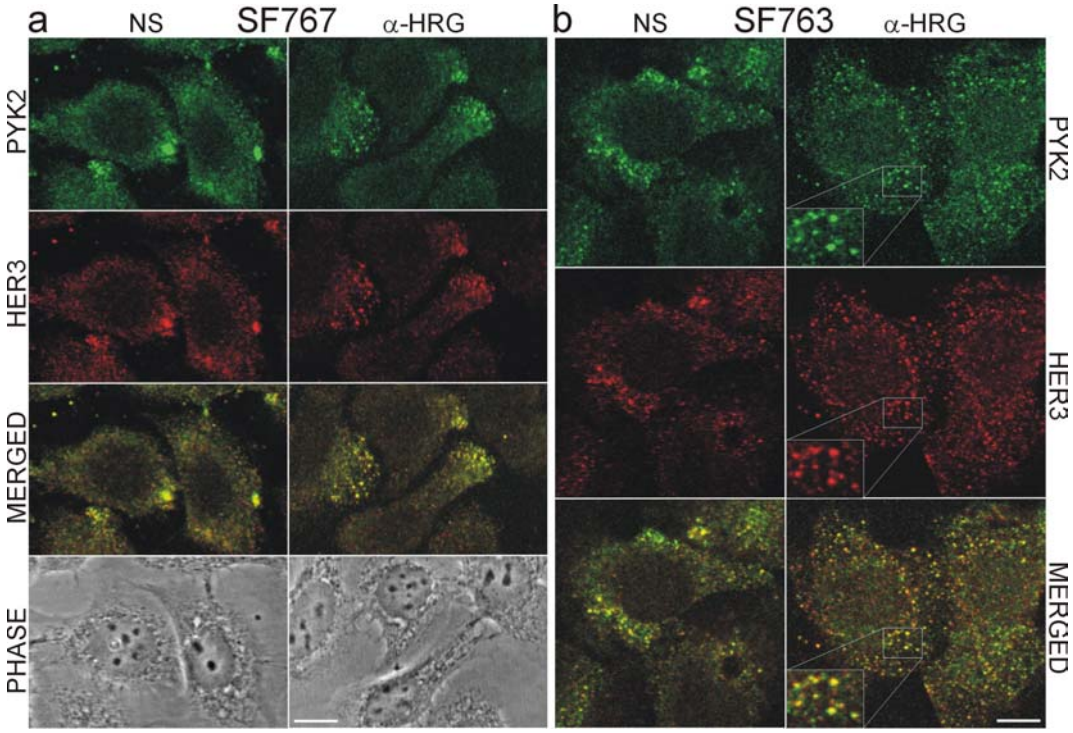

Fig. 9: **Localization of PYK2 and ErbB3/HER3 in SF763 and SF767 glioma cell lines. a, b,** In SF767 (**a**) and in SF763 cells (**b**), PYK2 shows a punctated distribution throughout the cytoplasm, and is enriched in the perinuclear region and in some prominent cell protrusions (green). ErbB3/HER3 (red) is largely colocalized, as shown by overlapping distributions of the two stains in most puncta (**b**, insets) and in larger aggregates (yellow). Colocalization is independent of stimulation by α-HRG. Cells were fixed and immunostained against PYK2 (green) and ErbB3/HER3 (red), either unstimulated (NS) or following stimulation with 5 µg ml^{-1} Heregulin for 20 min (α-HRG). Optical sections obtained by confocal laser scanning microscopy are shown. Scale bar represents 10 µm.

5.2. PYK2 associates with the intracellular region of ErbB3/HER3

To investigate in detail how tyrosine-phosphorylation of PYK2 depends on binding to ErbB3/HER3 we used an ectopic overexpression system. HEK293 fibroblasts were used to express either wild-type ErbB2/HER2, ErbB3/HER3 and PYK2 or dominant-negative mutant constructs ErbB2/HER2-KM, ErbB3/HER3-KM and PYK2-KM, where the lysine critical for ATP-binding was exchanged with alanine, rendering the kinase inactive. Tyrosine-phosphorylation of PYK2 was elevated upon HRG-stimulation of cells expressing all the wild-type constructs (Fig. 10a, lanes 1 and 2). However, in cells expressing ErbB3/HER3-KM (Fig. 10a, lanes 3 and 4) or ErbB2/HER2-KM (Fig. 10a, lanes 5 and 6), HRG-stimulation failed to induce PYK2 tyrosine-phosphorylation. This observation is consistent with the data from glioma cell lines (Fig. 8b), where inhibition of ErbB2/HER2 abrogated PYK2 activation, but further implies that HRG-induced PYK2 activation is dependent on functional kinase activities of both ErbB2/HER2 and ErbB3/HER3.

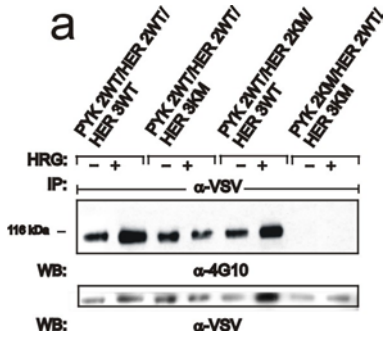

Fig. 10a: **Association of PYK2 with the C-terminal domain of ErbB3/HER3. a**, **b**, **c**, HEK293 fibroblasts were transfected with combinations of wild-type proteins (ErbB2/HER2, ErbB3/HER3, PYK2) and their dominant-negative variants (ErbB2/HER2-KM, ErbB3/HER3-KM, PYK2-KM). PYK2 was expressed tagged at its C-terminus with the vesicular somatitis virus glycoprotein (VSV). PYK2 tyrosine phosphorylation is dependent on functional ErbB2/HER2 and ErbB3/HER3 kinase activity. Cells were stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG), lysed and subjected to immunoprecipitation with monoclonal anti-VSV antibody (α-VSV). Immunocomplexes were analysed by western

blotting (WB) with a monoclonal anti-phosphotyrosine antibody (α-PY, upper panel). Equal loading of proteins was determined by reblotting with α-VSV antibody (lower panel).

Additionally, we used PYK2-KM to exclude a pronounced constitutive autophosphorylation of wild-type PYK2. Again, in cells expressing ErbB3/HER3-KM or ErbB2/HER2-KM, stimulation with HRG failed to induce PYK2 tyrosine-phosphorylation, strongly supporting our hypothesis that HRG-induced PYK2 activation is dependent on functional kinase activities of both ErbB2/HER2 and ErbB3/HER3 (Fig. 10b, lanes 5-8).

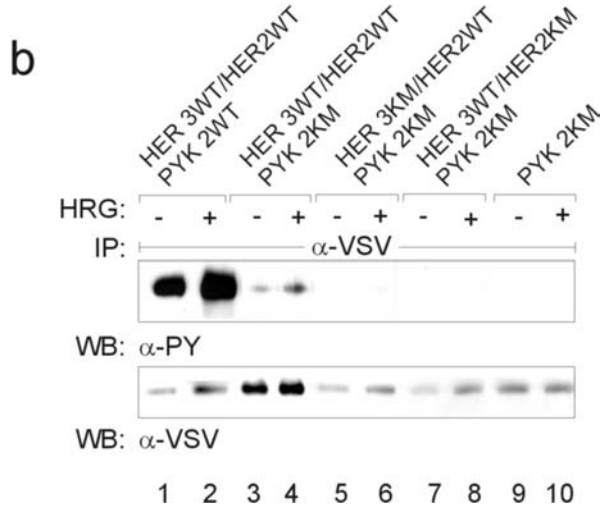

Fig. 10b: **Association of PYK2 with the C-terminal domain of ErbB3/HER3.** HEK293 fibroblasts were transfected with combinations of wild-type proteins (ErbB2/HER2, ErbB3/HER3, PYK2) and their dominant-negative variants (ErbB2/HER2-KM, ErbB3/HER3-KM). This experiment was performed in parallel to the experiment shown in Fig. 2a, using PYK2-WT instead of PYK2-KM. A prominent autophosphorylation of PYK2-WT is shown.

Next we used a mutant of ErbB3/HER3 lacking the C-terminal 697 amino acids (ErbB3/HER3ΔCT) to analyse the contribution of this domain to HRG-induced PYK2 tyrosine-phosphorylation (Fig. 10c). In PYK2 immunocomplexes of control cells expressing ErbB2/HER2 and ErbB3/HER3, we observed a coprecipitated protein of M_r=180 kDa which was phosphorylated and shown to be ErbB3/HER3 (Fig. 10c, lanes 1 and 2). The deletion mutant of ErbB3/HER3 abrogated tyrosine-phosphorylation of PYK2 and also the coprecipitation of ErbB3/HER3, indicating that PYK2 associates with the C-terminal region of ErbB3/HER3 (Fig. 10c, lanes 3 and 4). We also observed in cells expressing ErbB2/HER2 and ErbB3/HER3ΔCT an HRG-independent increase in tyrosine phosphorylation and protein expression of PYK2. This observation can be interpreted in that way that high levels of PYK2 expression leads to its constitutive tyrosine phosphorylation since overexpressed tyrosine kinases are capable to transphosphorylate each other through transient dimer formation even in the absence of a ligand.

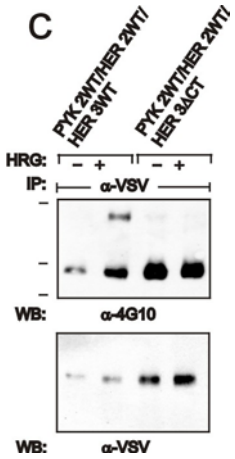

Fig. 10c: **Association of PYK2 with the C-terminal domain of ErbB3/HER3.** HEK293 fibroblasts were transfected with wild-type ErbB2/HER2 and PYK2 combined with wild-type ErbB3/HER3 or its truncated construct ErbB3/HER3ΔCT. Coprecipitating ErbB3/HER3 is indicated by an arrow. PYK2 was expressed tagged at its C-terminus with the vesicular somatitis virus glycoprotein (VSV). Cells were stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG), lysed and subjected to immunoprecipitation with monoclonal anti-VSV antibody (α-VSV). Immunocomplexes were analysed by western blotting (WB) with a monoclonal anti-phosphotyrosine antibody (α-PY, upper panel).

Equal loading of proteins was determined by reblotting with α-VSV antibody (lower panel).

The intracellular domain of ErbB3/HER3 harbours 13 phosphorylation sites that are thought to become transphosphorylated by ErbB2/HER2 after HRG-stimulation. Among these the tyrosines Y1035, Y1178, Y1203, Y1241, Y1257 and Y1270 are potential docking sites for the src-homology 2 (SH2) domains of the regulatory subunit p85 of PI_3-K (Hellyer et al., 1998), whereas Y1309 is a binding site for SHC (Prigent and Gullick, 1994). To identify the specific binding sites for PYK2 in the C-terminal domain of ErbB3/HER3, we used 13 add-back mutants, in which all tyrosine residues had been replaced by phenylalanines with the exception of one. We performed transfection experiments in HEK293 fibroblasts, using wildtype PYK2 and ErbB2/HER2, and single add-back mutants of ErbB3/HER3 (Fig. 10d). Using this approach, we identified three tyrosine residues Y1257, Y1270 and Y1288, which are critical for elevated PYK2 tyrosine-phosphorylation upon HRG-stimulation (Fig. 10d, lanes 13-18) and its physical association with HER3 (Fig. 10e, lanes 5-10). Based on these observations we conclude that the HRG-induced stimulation of PYK2 tyrosine-phosphorylation depends on its binding to Y1257, Y1270 and Y1288 in the C-terminal domain of ErbB3/HER3.

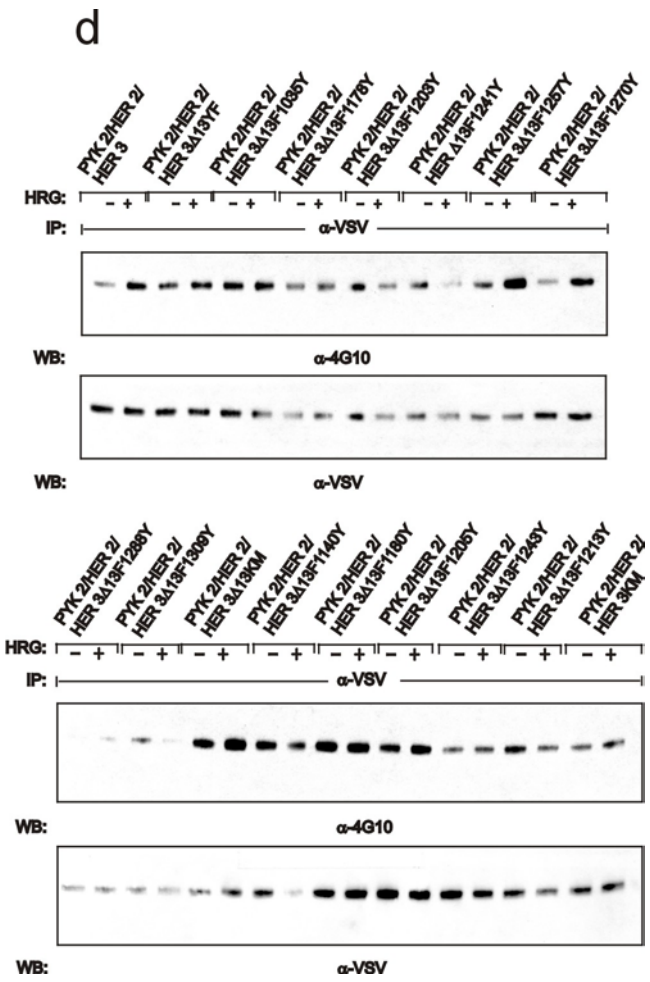

Fig. 10d: **Association of PYK2 with the C-terminal domain of ErbB3/HER3.** HEK293 fibroblasts were transfected as indicated. PYK2 activation is dependent on Y1257, Y1270 and Y1288 in the C-terminal domain of ErbB3/HER3. PYK2 was expressed tagged at its C-terminus with the vesicular somatitis virus glycoprotein (VSV). Cells were stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG), lysed and subjected to immunoprecipitation with monoclonal anti-VSV antibody (α-VSV). Immunocomplexes were analysed by western blotting (WB) with a monoclonal anti-phosphotyrosine antibody (α-PY, upper panel). Equal loading of proteins was determined by reblotting with α-VSV antibody (lower panel).

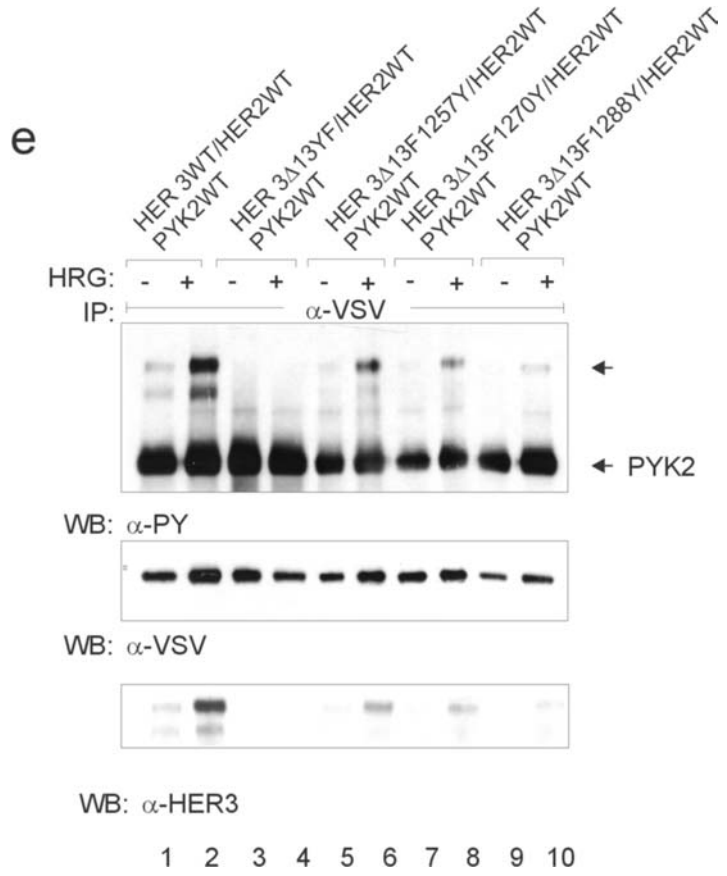

Fig. 10e: **Association of PYK2 with the C-terminal domain of ErbB3/HER3.** HEK293 fibroblasts were transfected as indicated. PYK2 activation is dependent on Y1257, Y1270 and Y1288 in the C-terminal domain of ErbB3/HER3. PYK2 was expressed tagged at its C-terminus with the vesicular somatitis virus glycoprotein (VSV). Cells were stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG), lysed and subjected to immunoprecipitation with monoclonal anti-VSV antibody (α-VSV). Immunocomplexes were analysed by western blotting (WB) with a monoclonal anti-phosphotyrosine antibody (α-PY, upper panel). Note that ovexposure was required to detect coprecipitated HER3 in PYK2-VSV immunoprecipitates. PYK2 activation is dependent on Y1257, Y1270 and Y1288 in the C-terminal domain of HER3. Equal loading of proteins was determined by reblotting with α-VSV antibody (lower panel).

5.3. Tyrosine-phosphorylation of PYK2 is dependent on ErbB3/HER3 kinase activity

Although ErbB3/HER3 can bind ATP and its analog TNP-ATP (Sierke et al., 1997), it is widely believed that its kinase is inactive (Guy et al., 1994). This is based on the fact that two tyrosine kinase consensus positions in ErbB3/HER3 differ from other members of this gene family and experimental data with HER3 protein that indicated much lower kinase activity than that of the EGFR (Sierke et al., 1997). To identify the kinase which is responsible for the PYK2 tyrosine-phosphorylation upon HRG-stimulation, we conducted *in vitro* kinase assays with myelin basic protein (MBP) and a GST-fusion protein of the C-terminal region of PYK2 (GST-PYK2-CT) as substrates, in either anti-ErbB2/HER2 or anti-ErbB3/HER3 immunoprecipitates (Fig. 11a).

Upon stimulation of SF767 cells either with HRG, or with Phorbol-12-myristate-13-acetate (PMA), MBP became phosphorylated by HER2, but not by ErbB3/HER3 (Fig. 11a, white bars). Surprisingly, however, GST-PYK2-CT became phosphorylated by ErbB3/HER3 in a HRG-stimulation-dependent way, but not by ErbB2/HER2 (Fig. 11a, black bars).

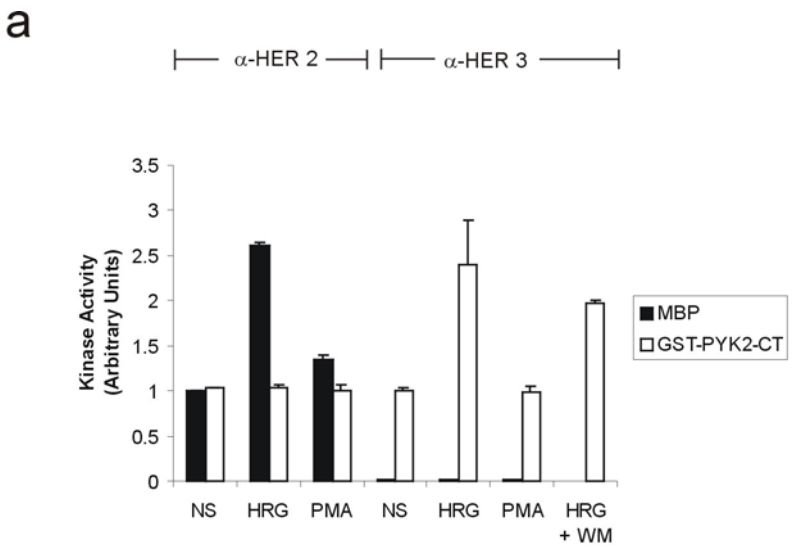

Fig. 11a: **Phosphorylation of GST-PYK2-CT by ErbB3/HER3 upon HRG stimulation. a**, **b**, SF767 gliomas were either stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG) or with 1 μM Phorbol-12-myristate-13-acetate for 10 min (PMA). PMA stimulation was

used as a negative control. Note that kinase activity of ErbB3/HER3 is under 1% of the corresponding HER2 activity when using MBP as a substrate, in contrast to GST-PYK2-CT.

As it has been shown that PI_3-K binds to the cytoplasmic tail of ErbB3/HER3, we investigated a potential role of PI_3-K in PYK2 phosphorylation by precipitating either ErbB2/HER2 or ErbB3/HER3 in the presence or absence of the PI_3-K-inhibitor Wortmannin (WT) (Fig. 11b). The results indicate that PI_3-K is not involved in the direct phosphorylation of GST-PYK2-CT. Consistent with this finding, precipitation of PYK2 under the same experimental conditions showed that its elevated tyrosine-phosphorylation upon HRG-stimulation is independent of PI_3-K (data not shown). Taken together, these data suggest that the ErbB3/HER3 kinase is active and phosphorylates tyrosine residues in the C-terminal region of PYK2.

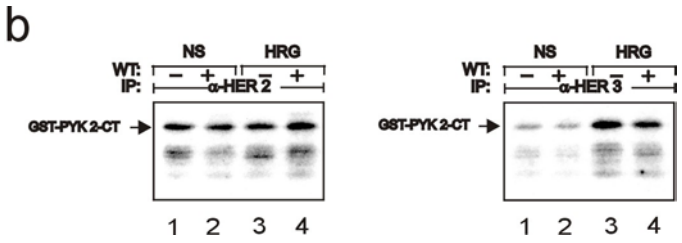

Fig. 11b: **Phosphorylation of GST-PYK2-CT by ErbB3/HER3 upon HRG stimulation.** SF767 gliomas were either stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG) or were pretreated with 100 nM Wortmannin for 30 min (WT). Upon α-HRG stimulation, phosphorylation of GST-PYK2-CT by ErbB3/HER3 is upregulated, in contrast to ErbB2/HER2 activity. Influence of WT is negligible, thus excluding involvement of PI_3-K in PYK2 phosphorylation. Phosphorylated GST-PYK2-CT is indicated by an arrow.

To verify that ErbB3/HER3 directly phosphorylates PYK2, we overexpressed ErbB2/HER2 and ErbB3/HER3 either separately, or together in combinations with dominant-negative mutants in HEK293 cells (Fig. 11c). Receptor-immunocomplexes were subjected to *in vitro* kinase assays and revealed that, after HRG-stimulation, GST-PYK2-CT is phosphorylated in ErbB3/HER3 but not in immunoprecipitates (Fig. 11c, upper panel, lanes 3, 4, 5 and 6 vs. lanes 1 and 2).

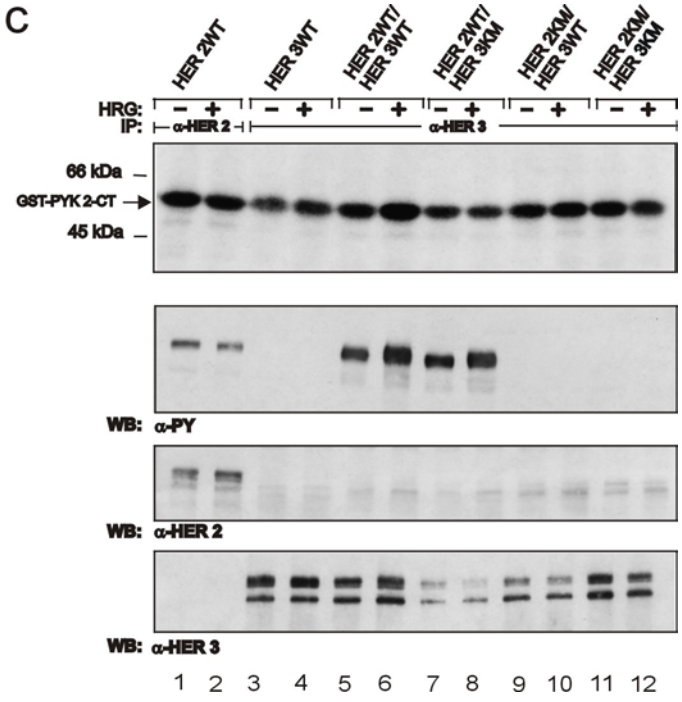

Fig. 11c: **Phosphorylation of GST-PYK2-CT by ErbB3/HER3 upon HRG stimulation.** HEK293 fibroblasts were transfected with the combinations of wild-type proteins (ErbB2/HER2, ErbB3/HER3) and their dominant-negative variants (ErbB2/HER2-KM, ErbB3/HER3-KM) as indicated, and stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG). Only homodimers of ErbB3/HER3 and heterodimers of ErbB3/HER3 with ErbB2/HER2 induced an increased GST-PYK2-CT phosphorylation (**c**, upper panel). Heterodimerization of ErbB3/HER3 with leads to a stronger phosphorylation of the substrate, indicating that ErbB2/HER2 is important for ErbB3/HER3 activation. Transphosphorylation of ErbB3/HER3 by ErbB2/HER2 was checked by probing the membrane with an anti-phosphotyrosine antibody α-PY (**c**, upper middle panel). Coprecipitation of ErbB2/HER2 with ErbB3/HER3 was excluded by probing the membrane with anti- antibody α- (**c**, lower middle panel). Equal loading of proteins was

checked by probing with anti-ErbB3/HER3 antibody (α-ErbB3/HER3) (**c**, lower panel). Phosphorylated GST-PYK2-CT is indicated by an arrow.

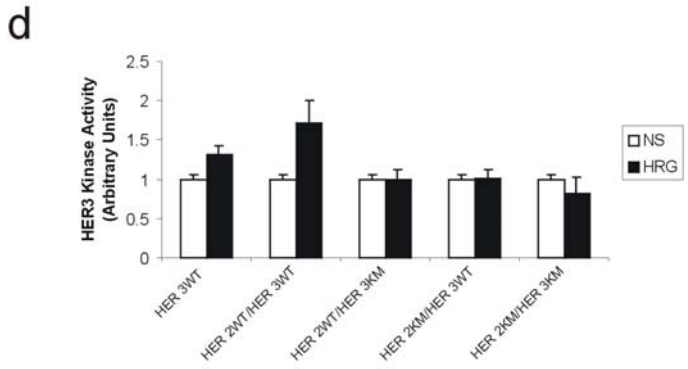

Fig. 11d: **Phosphorylation of GST-PYK2-CT by ErbB3/HER3 upon HRG stimulation.** Quantification of the kinase activity shown in the upper panel of Fig. 11c.

ErbB3/HER3 homodimers also phosphorylated GST-PYK2-CT, but to a lesser extent compared to transactivated ErbB3/HER3 (Fig. 11c, upper panel, lanes 3 and 4). To show that ErbB3/HER3 is transphosphorylated by upon HRG-stimulation, we probed the blot with the monoclonal anti-phosphotyrosine antibody 4G10 (Fig. 11c, middle upper panel). We also show that there was no significant coprecipitation of ErbB2/HER2 in the ErbB3/HER3 immunocomplex under our assay conditions, strongly suggesting that ErbB2/HER2 is not the kinase, which phosphorylates GST-PYK2-CT (Fig. 11c, middle lower panel). We conclude that ErbB3/HER3 directly phosphorylates PYK2 upon HRG-stimulation.

Additionally, we determined on which amino acid residues the phosphorylation event of GST-PYK2-CT occurred. We detected phosphorylated tyrosine and serine residues but not threonine residues (Fig. 11e, compare upper middle panel with middle and lower panels). Phosphorylation of tyrosine residues was dependent on HRG stimulation, whereas phosphorylation of serine residues was constitutive and independent of HRG stimulation, suggesting that a serine kinase coprecipitated with HER3. The findings that the HER2 kinase is not able to phosphorylate GST-PYK2-CT in a HRG dependent manner, and that the

exchange of a single methionine to lysine in HER3 prevents GST-PYK2-CT phosphorylation, clearly indicate that HER3 is an active kinase responsible for the phosphorylation of tyrosine residues in the C-terminal region of PYK2.

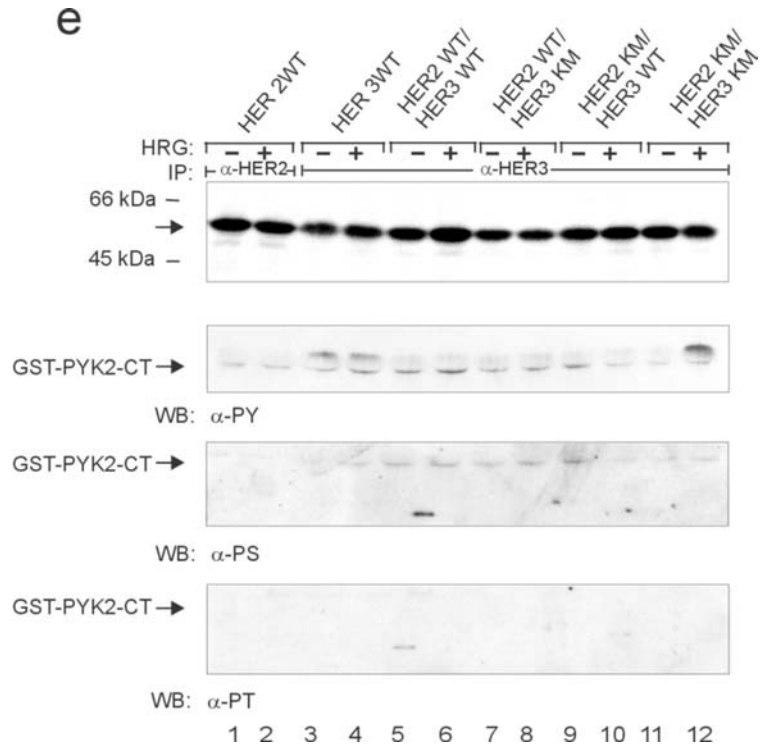

Fig. 11e: **Phosphorylation of GST-PYK2-CT on tyrosine and serine, but not on threoinine residues upon HRG stimulation.** In order to elucidate the phosphorylation content of GST-PYK2-CT, the blot was probed either with phosphotyrosine (α-PY), phosphoserine (α-PS) or phosphothreonine (α-PT) antibody. GST-PYK2-CT becomes tyrosine phosphorylated upon HRG stimulation, whereas constitutive serine phosphorylation and no threonine phosphorylation, respectively, is detectable upon HRG stimulation. This is a representative kinase assay of the experiment shown in Fig. 11d.

5.4. *In vitro* phosphorylation of GST-PYK2-CT by recombinant purified GST-HER3-KD

To exclude the possibility that an associating tyrosine kinase phosphorylates GST-PYK2-CT in our mammalian systems, we purified bacterially expressed recombinant GST-fusion proteins of the HER2 (GST-HER2-KD) and HER3 (GST-HER3-KD) kinase domains. We performed *in vitro* kinase assays using either GST-HER2-KD, GST-HER3-KD or recombinant c-SRC as enzymes and GST-PYK2-CT as substrate (Fig. 12a). While recombinant c-SRC showed the strongest phosphorylation of GST-PYK2-CT (Fig. 12a left panel, lane 1), we also observed significant phosphorylation of GST-PYK2-CT by GST-HER2-KD, as well as GST-HER3-KD (Fig. 12a, left panel, compare lanes 2 and 3). To show specificity of the kinase reaction we repeated the experiment using MBP as substrate (Fig. 12b left panel). Again strong phosphorylation by c-SRC was observed (Fig. 12b, left panel, lane 1), a weaker one by GST-HER2-KD, but no phosphorylation by GST-HER3-KD (Fig. 12b, left panel, compare lanes 2 and 3). This experiment clearly demonstrated that in a defined *in vitro* system HER3 has kinase activity and GST-PYK2-CT serves as substrate for the enzymatic function of HER3. It further confirms and substantiates the data obtained with intact SF767 and HEK293 cells. The fact that HER2 was able to phosphorylate GST-PYK2-CT *in vitro*, while this was not the case HRG-dependently in intact cells suggest the involvement of cellular parameters in substrate selection.

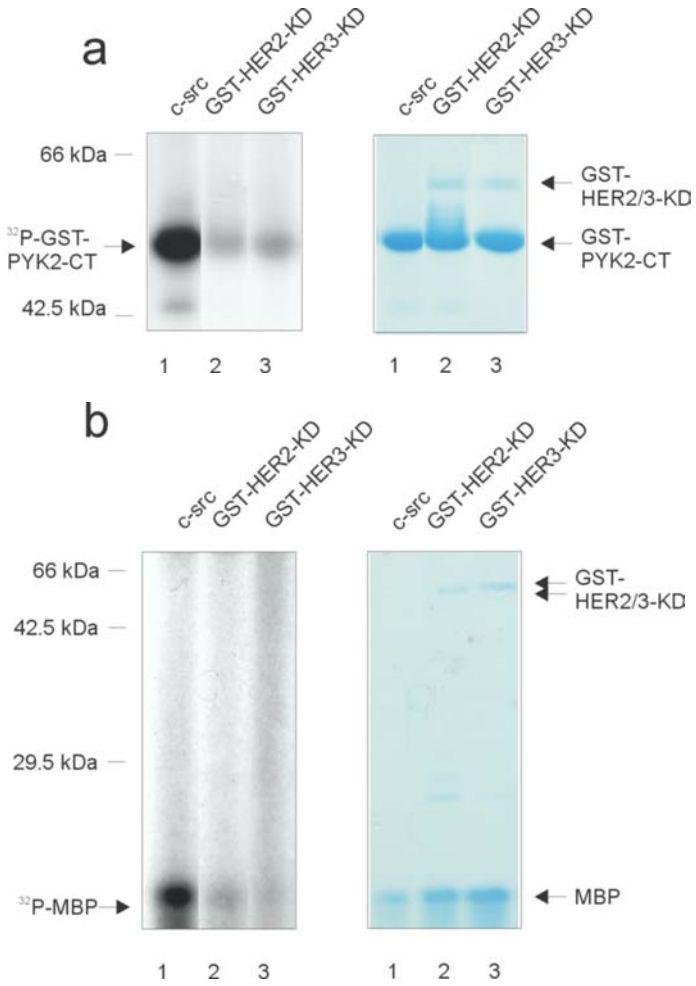

Fig. 12: **GST-HER3-KD tyrosine phosphorylates GST-PYK2-CT**. (**a**) Recombinant c-SRC, and bacterially expressed GST-HER2-KD and GST-HER3-KD were used as enzymes and GST-PYK2-CT (**a**) as substrate. Coomassie-stained gels are shown to confirm equal protein loading (**a**, right panel). (**b**) The same experimental procedure was used as in (**a**), except that MBP was used as the substrate. Note that GST-HER3-KD phosphorylates GST-PYK2-CT stronger than GST-HER2-KD, whereas using MBP as substrate no phosphorylation of MBP over background occurred, demonstrating substrate specificity of HER3.

5.5. PYK2 amplifies the ErbB2/HER2-ErbB3/HER3 mitogenic signal

Upon stimulation of ErbB3/HER3 and ErbB2/HER2, PI_3-K and SHC bind to the C-terminus of ErbB3/HER3 and mediate the transmission of a signal through the Ras/Raf pathway (Yarden and Sliwkowski, 2001). To test the influence of ErbB2/HER2 and PI_3-K on MAPK activation, we exposed SF767 cells to respective inhibitors AG825 and Wortmannin (WT) prior to stimulation with HRG. Then we precipitated SHC or performed MAP-kinase assays (Fig. 13a, b).

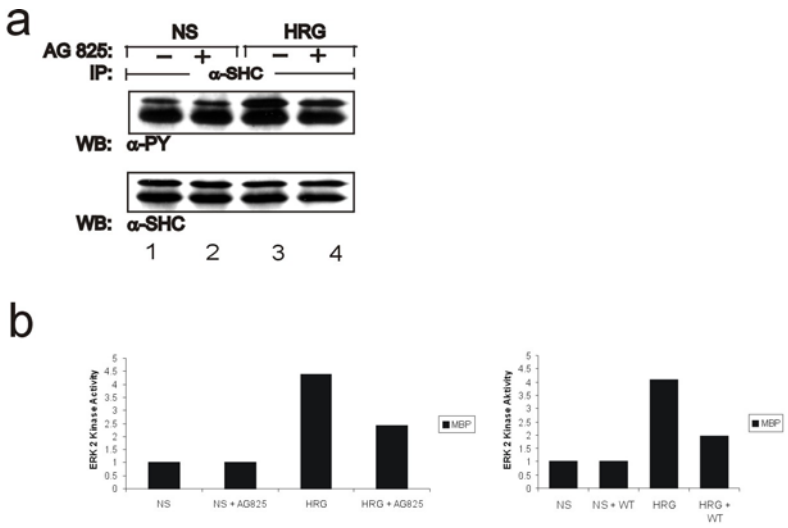

Fig. 13a, b: **PYK2 mediates a mitogenic response upon HRG stimulation. a**, **b**, SF767 gliomas were pretreated either with 10 μM AG825 for 1 hour or with 100 nM Wortmannin for 30 min (WT), and then stimulated with 5 μg ml^{-1} Heregulin for 20 min (α-HRG). Tyrosine phosphorylation of SHC was elevated by α-HRG and attenuated by pretreatment with AG825, but not fully abrogated (**a**). The same holds also for ERK-2 activity, when cells were pretreated either with AG825 or with WT (**b**). Cell lysates were used for immunoprecipitation with polyclonal anti-SHC (α-SHC) (**a**), or polyclonal anti-Erk-2 (α-ERK-2) antibodies (**b**). α-SHC-immunocomplexes were blotted with a monoclonal anti-phosphotyrosine antibody (α-PY) (**a**), whereas α-ERK-2 immunocomplexes were subjected to MAP-kinase assays (**b**). Phosphorylated MBP is indicated by an arrow. Phosphorylated MBP is indicated by an arrow.

HRG-stimulated tyrosine-phosphorylation of SHC and ERK-2 activity was diminished, but not fully abrogated by inhibition of ErbB2/HER2 (Fig. 13a, 13b, left panel). The analogous experiment using WT for inhibition of PI_3-K revealed that ERK-2 activity was reduced by WT (Fig. 13b, right panel). These findings indicate that the HRG-induced MAPK signal only partially depends on ErbB2/HER2 and PI_3-K.

To characterize in more detail the role of PYK2 downstream of the ErbB2/HER2-ErbB3/HER3 signal, we used a tetracyclin-inducible system (Tet-off) in the pheochromocytoma cell line PC12 (Zwick et al., 1999). PC12 cells are rich in PYK2, so that in the presence of Tet only endogenous PYK2 is expressed, whereas Tet removal leads to overexpression of kinase-deficient dominant-negative PYK2 (PYK2-KM). We inhibited either ErbB2/HER2 or PI_3-K with AG825 and WT, respectively, prior to stimulation with HRG, precipitated ERK-2 and subjected the immunocomplexes to MAP-kinase assays (Fig. 13c).

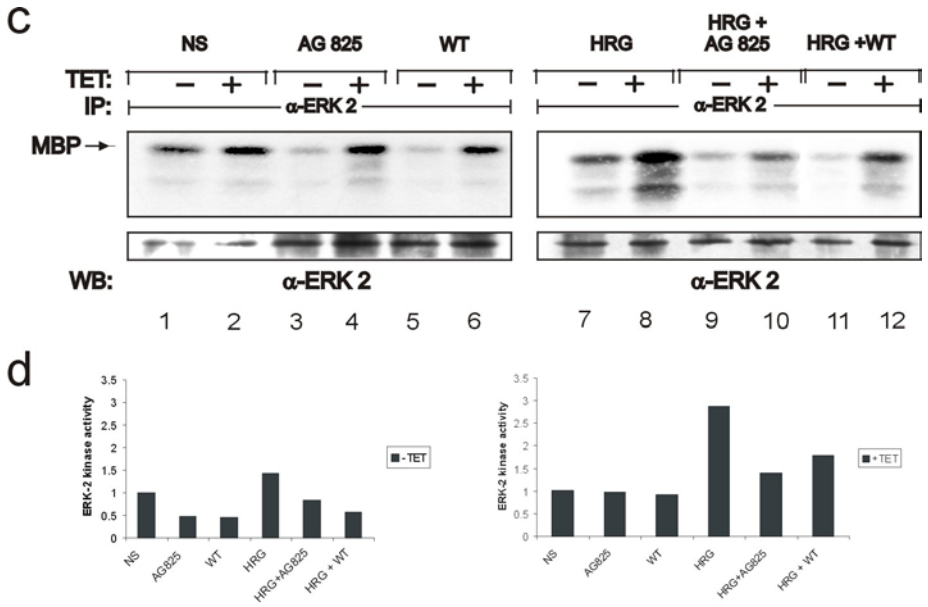

Fig. 13c, d: **PYK2 mediates a mitogenic response upon HRG stimulation. c**, Tetracyclin-inducible pheochromocytoma PC12 cells, either stably expressing PYK2-KM (Tet-), or only endogenous PYK2 (Tet+), were pretreated either with 100 nm Wortmannin for 30 min (WT), or 10 μM AG825 for 1 hour prior to stimulation with 5 μg ml^{-1} Heregulin for 20 min (α-HRG). Basal ERK-2 activity is independent of ErbB2/HER2 and PI_3-K, whereas the α-HRG-stimulated ERK-2 activity is dependent on ErbB2/HER2,PI_3-K and

PYK2. Overexpression of PYK2-KM leads to a general attenuation of ERK-2 activity (compare Tet- with Tet+ bands). Equal loading of proteins was checked by probing with anti-ERK-2 antibody (α-ERK-2). Phosphorylated MBP is indicated by an arrow. **d**, Quantification of the ERK-2 kinase activity shown in Fig. 13c.

Basal ERK-2 activity was not influenced by AG825 and WT, but was abrogated upon PYK2-KM expression. HRG-stimulated ERK-2 activity, however, was attenuated by the two inhibitors, and also abrogated by PYK2-KM expression. These findings are consistent with the results obtained in SF767 (Fig. 13b), and together indicate that the constitutive basal ERK-2 activity in both PC12 and SF767 glioma cells depends on PYK2, and is independent of ErbB2/HER2 and PI_3-K, whereas further stimulation of ERK-2 by HRG requires ErbB2/HER2, PI_3-K, and PYK2.

5.6. Involvement of PYK2 in ErbB2/HER2-ErbB3/HER3-mediated tumour invasion

In addition to its role in cell proliferation and anti-apoptosis, an involvement of PI_3-K in carcinoma invasion has recently been demonstrated (Shaw et al., 1997). We therefore investigated the potential of PYK2 and its dominant-negative mutant PYK2-KM to influence PI_3-K activation upon HRG-stimulation. Using the Tet-off system in PC12 cells, we subjected cell lysates to PI_3-K assays, where we observed PYK2-dependent PI_3-K activation upon HRG-stimulation (Fig. 14, upper panel, lanes 1 vs. 2 with 7 and 8).

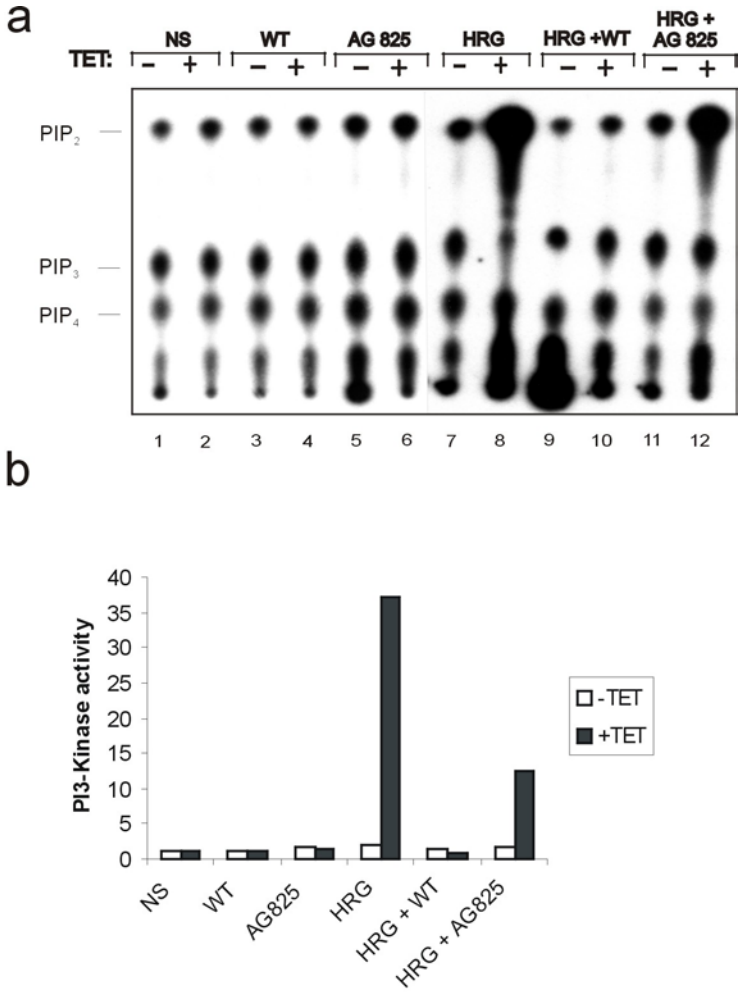

Fig. 14: **PYK2 enhances PI_3-K activity upon HRG stimulation. a**, Tetracyclin-inducible pheochromocytoma PC12 cells, either stably expressing PYK2-KM (Tet-) or only endogenous PYK2 (Tet+), were pretreated either with 100 nm Wortmannin for 30 min (WT), or 10 μM AG825 for 1 hour prior to stimulation with 5 μg ml^{-1} Heregulin for 20 min (α-HRG). Lysates were subjected to α-PY immunoprecipitation and PI_3-K assays were performed (see Methods section). PI_3-K activity is strongly dependent on PYK2 upon α-HRG stimulation, and is diminished by AG825. Phosphorylated Phosphatidylinositol is indicated. **b**, Quantification of the PI_3-K kinase activity shown in Fig. 14a.

Inhibition of ErbB2/HER2 kinase activity did only partially abrogate PI_3-K activity, indicating the involvement of a ErbB2/HER2-independent mechanism of PI_3-K activation (Fig. 14, upper panel, lanes 8 and 12) and an important role of PYK2 in HRG induction of MAPK signalling and in PI_3-K activation.

Further experiments adressed the role of PYK2 in the invasive properties of glioma cells. Gliomas represent highly malignant brain tumours with a very poor prognosis (Berens and Giese, 1999). It has been shown that PI_3-K links $\alpha6\beta4$-integrin signalling to invasive behaviour of breast tumour cells (Shaw et al., 1997). Furthermore, it has been reported that activation of MAPK through $\alpha6\beta4$-integrin signalling is relevant to invasion, due to its importance in migration and its ability to phosphorylate myosin light chain kinase (Klemke et al., 1997). Using the C6 glioma cell line as a model system for tumour invasion (Kaye et al., 1986), we tested whether PYK2 activity is required for this process and if so, by what mechanism. We used a recombinant retrovirus to transfer coding sequences for PYK2-KM into C6 glioma cells prior to stimulation with HRG. To ask whether the MAPK pathway plays a role in the process, we also pretreated the cells with the MEK1 inhibitor PD98059 (Fig. 15a).

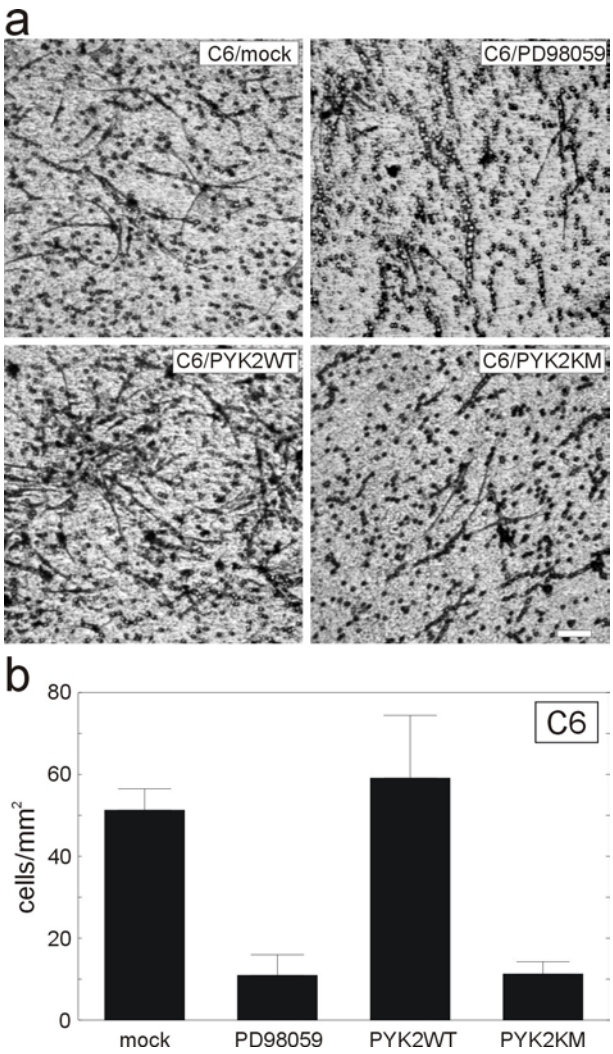

Fig. 15a, b: **PYK2-KM inhibits tumour invasion upon HRG stimulation. a**, C6 gliomas were retrovirally infected with either a control vector pLXSN (mock), PYK2, dominant negative PYK2 mutant PYK2-KM, or pretreated with a MEK1 inhibitor PD98059 (25 μM) for 30 min, and tumour invasion assays were performed (see Methods section). **b**, Invasion is supressed to the same extent by PD98059 and by overexpression of PYK2-KM ($p>0.95$). Representative bright-field micrographs of cells that migrated through the 8 μm filters in 16 h are shown. Scale bars represent 100 μm (**a**).

MEK1-inhibition strongly attenuated invasiveness and a comparable abrogation of the invasive phenotype was observed upon infection of cells with PYK2-KM. The mitogenic signal of the ErbB2/HER2-ErbB3/HER3 dimer seems to be downregulated by PYK2-KM, however, overexpression of PYK2 in C6 cells did not significantly enhance their invasive phenotype (Fig. 15b). Endogenous PYK2 expression in C6 cells is comparably weaker than in SF763 or SF767 cells (data not shown), but this does not seem to interfere with their invasive potency. We also tested glioma cell lines SF763 and SF767 in the tumour invasion assay, after viral infection with the PYK2-KM construct. Again, a strong inhibition of the invasive behaviour of tumour cells by PYK2-KM was observed (Fig. 15d). These results demonstrate that PYK2 can activate the MAPK pathway, which plays an important role in glioma invasion upon HRG-stimulation.

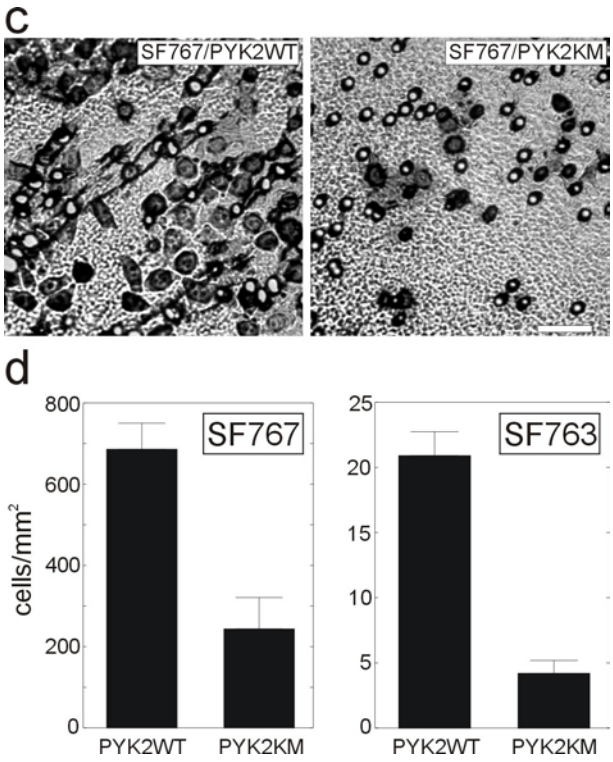

Fig. 15c, d: **PYK2-KM inhibits tumour invasion upon HRG stimulation. c**, SF767 gliomas were retrovirally infected with pLXSN or PYK2-KM. **d**, Tumor invasion is supressed by overexpresssion of PYK2-KM in SF767 ($p<0.008$), and also in SF763 cell line ($p<0.005$), as shown by using the same assay as in Fig 15a, b. Representative

bright-field micrographs of cells that migrated through the 8 μm filters in 16 h are shown. Scale bars represent 50 μm (**c**).

In parallel we examined ERK-2 phosphorylation events and determined a decrease in ERK-2 phosphorylation by using either PD98059 or expressing PYK2-KM in both C6 and SF767 cell lines (Fig. 15e, f upper panel, compare lanes 6 and 7 with 5). These results indicate that PYK2 activates the MAPK pathway, and thereby plays a critical role in HRG-mediated glioma invasion.

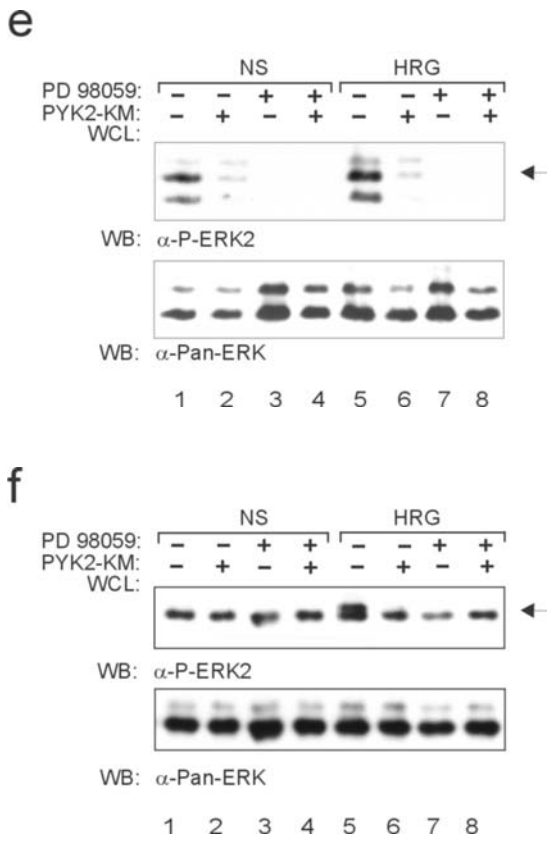

Fig. 15e, f: **PYK2-KM inhibits ERK-2 activity in C6 and SF767 cells.** (**e**) Whole cell lysates (WCL) of C6 glioma cells were prepared in parallel to the tumor invasion assay and the content of phosphorylated ERK-2 was assessed by probing with a specific phospho-ERK-2 antibody (upper panel). To confirm equal loading of proteins the blot was reprobed with a pan-ERK antibody. (**f**) The same experimental procedure was used as in (**e**) for SF767 cells. Phosphorylated ERK-2 is indicated by an arrow. Dominant-negative PYK2-KM abrogates ERK-2 activity to the same extent as MEK-1 inhibitor PD98059.

5.7. α-HER3^{ECD} interferes with receptor tyrosine phosphorylation and dimerisation of ErbB3/HER3 and ErbB2/HER2

We have compared the effects of a monoclonal antibody against the extracellular domain of ErbB3/HER3 with those of the anti-ErbB2/HER2 therapeutic monoclonal antibody Herceptin® (HC) on HRG-mediated signalling. The breast cancer cell lines MCF-7^{ADR} and MDA-MB-468 were chosen as a model on the basis of their different ratios of ErbB2/HER2:ErbB3/HER3 and their inherent migratory properties, with MDA-MB-468 being the most invasive cell line. The ratios of ErbB2/HER2:ErbB3/HER3 were about 1:3 and 3:1 in MDA-MB-468 and MCF-7^{ADR} respectively, based on cDNA array analysis data and western blot (E.H.v.d.H. unpublished observation). In order to assess the effect of α-HER3^{ECD} in comparison to HC, the cells were pretreated with α-HER3^{ECD} or HC respectively prior to HRG stimulation. Receptor-immunoprecipitation was then performed and the phosphotyrosine content of ErbB2/HER2 and ErbB3/HER3 analyzed by western blot with an anti-phosphotyrosine (PY) antibody (Fig. 16).

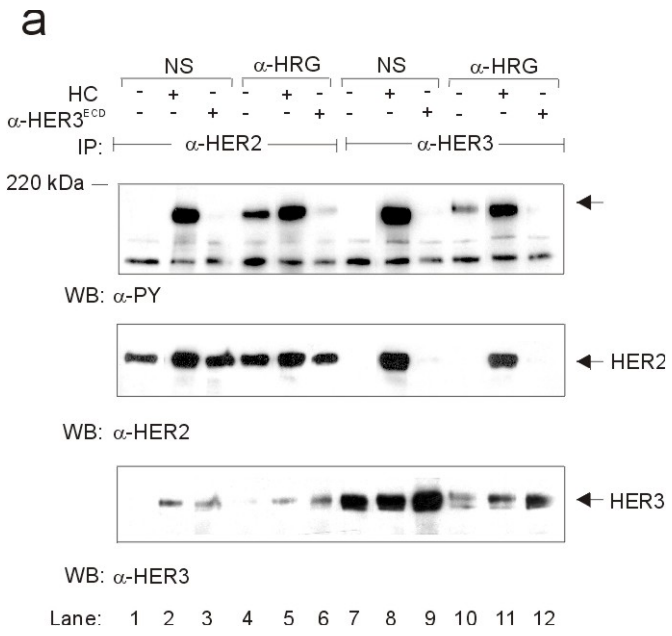

Fig. 16a: **α-HER3^{ECD} interferes with receptor tyrosine phosphorylation and dimerisation of ErbB3/HER3 and ErbB2/HER2 in MCF-7^{ADR} cells . a**, Serum starved breast cancer cells MCF-7^{ADR} were pretreated either with 50 μg/ml HC or 10 μg/ml α-HER3^{ECD} for 60 minutes. Cell stimulation was carried out for 5 min. with 5 μg ml^{-1} α-Heregulin (HRG) for MCF-7^{ADR} . Cell lysates were subjected to immunoprecipitation (IP) using monoclonal anti-ErbB2/HER2 (α-ErbB2/HER2) or monoclonal anti-ErbB3/HER3 (α-ErbB3/HER3) antibodies. The level of tyrosine phosphorylation was analyzed by western blotting (WB) with a monoclonal anti-PY antibody (α-4G10) (upper panels). Reblotting with α-ErbB2/HER2 and α-ErbB3/HER3 antibodies shows equal protein loading (middle and bottom panels). Unstimulated cells are indicated by NS. Coprecipitating proteins are indicated by an arrow.

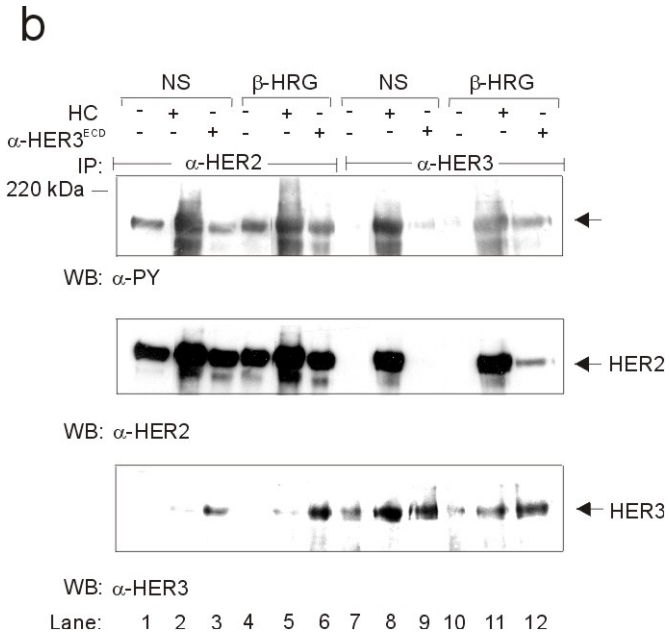

Fig. 16b: **α-HER3^{ECD} interferes with receptor tyrosine phosphorylation and dimerisation of ErbB3/HER3 and ErbB2/HER2 in MDA-MB-468. b,** Serum starved breast cancer cells MDA-MB-468 were pretreated either with 50 μg/ml HC or 10 μg/ml α-HER3^{ECD} for 60 minutes. Cell stimulation was carried out for 5 min. with 10 ng/ml β-Heregulin for MDA-MB-468. Cell lysates were subjected to immunoprecipitation (IP) using monoclonal anti-ErbB2/HER2 (α-ErbB2/HER2) or monoclonal anti-ErbB3/HER3 (α-ErbB3/HER3) antibodies. The level of tyrosine phosphorylation was analyzed by western blotting (WB) with a monoclonal anti-PY antibody (α-4G10) (upper panels). Reblotting with α-ErbB2/HER2 and α-ErbB3/HER3 antibodies shows equal protein loading (middle and bottom panels). Unstimulated cells are indicated by NS. Coprecipitating proteins are indicated by an arrow.

As shown in figure 16, pretreatment with α-HER3^{ECD} substantially decreased the tyrosine phosphorylation level of ErbB3/HER3 and ErbB2/HER2 after α-HRG stimulation in MCF-7 (Fig. 16a); in MDA-MB-468, where ErbB2/HER2 is more abundant than ErbB3/HER3, the tyrosine phosphorylation of ErbB2/HER2 was still reduced whilst, interestingly, an increase of ErbB3/HER3 tyrosine phosphorylation and of ErbB2/HER2-ErbB3/HER3 association could be observed (Fig. 16b, lanes 6 and 12). In contrast, HC upregulated receptor tyrosyl-phosphorylation and promoted association of ErbB3/HER3 and ErbB2/HER2, independent of HRG stimulation, in both cell lines (Fig. 16a, b).

5.8. α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of SHC and PI_3-K with ErbB3/HER3

We subsequently asked whether α-HER3^{ECD} has an effect on the known substrates of ErbB3/HER3, namely SHC and PI_3-K, which are effector proteins responsible for MAPK cascade activation and lipid signalling respectively (Wallasch et al., 1995; Yarden and Sliwkowski, 2001). To address this question, we immunoprecipitated SHC and the regulatory subunit of PI_3-K, p85, under the experimental conditions described above and assessed the tyrosine phosphorylation of these effectors (Fig. 17).

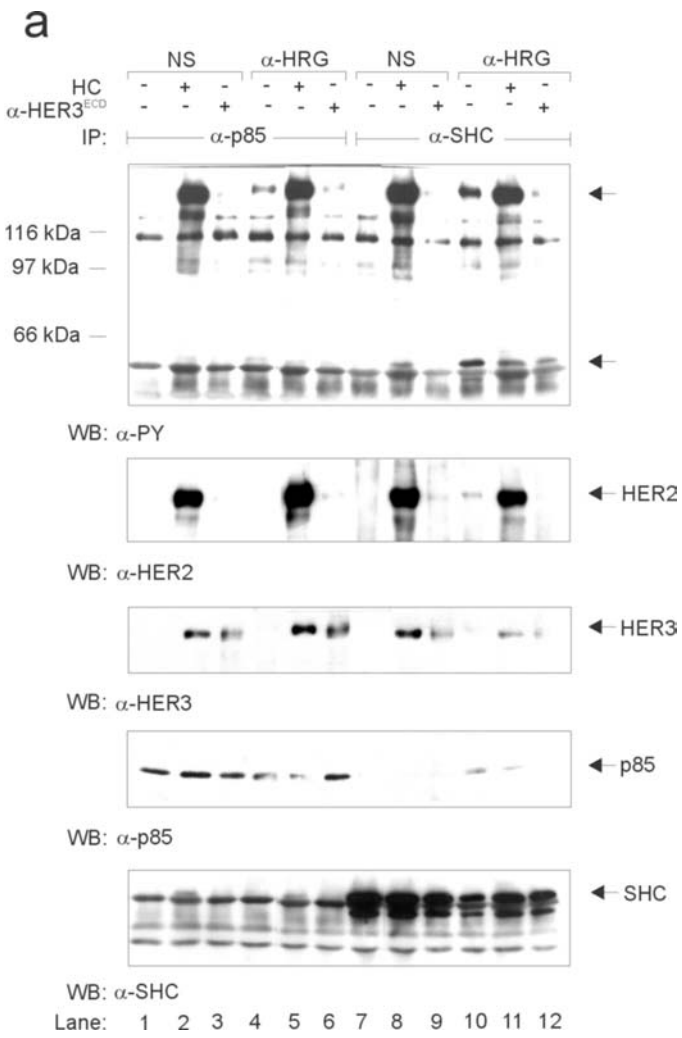

Fig. 17a: **α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of SHC and PI$_3$-K with ErbB3/HER3 in MCF-7^{ADR} cells.** Serum starved breast cancer cells MCF-7^{ADR} were pretreated and stimulated as described in figure 16. Cell lysates were either subjected to immunoprecipitation (IP) using monoclonal anti-SHC2 (α-SHC2) or monoclonal anti-PI$_3$-K (α-PI$_3$-K) antibodies. Tyrosine phosphorylation levels were analyzed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-4G10) (**a**, upper panels). Reblots with α-SHC and α- PI$_3$-K antibodies were performed as a control for protein loading (**a**, bottom panels). Coprecipitation of ErbB2/HER2 and ErbB3/HER3 with SHC or with PI$_3$-K was detected by probing the membrane either with α-ErbB2/HER2 or α- ErbB3/HER3 antibody (**a**, middle panes). Unstimulated cells are indicated by NS. Coprecipitating proteins are indicated by an arrow.

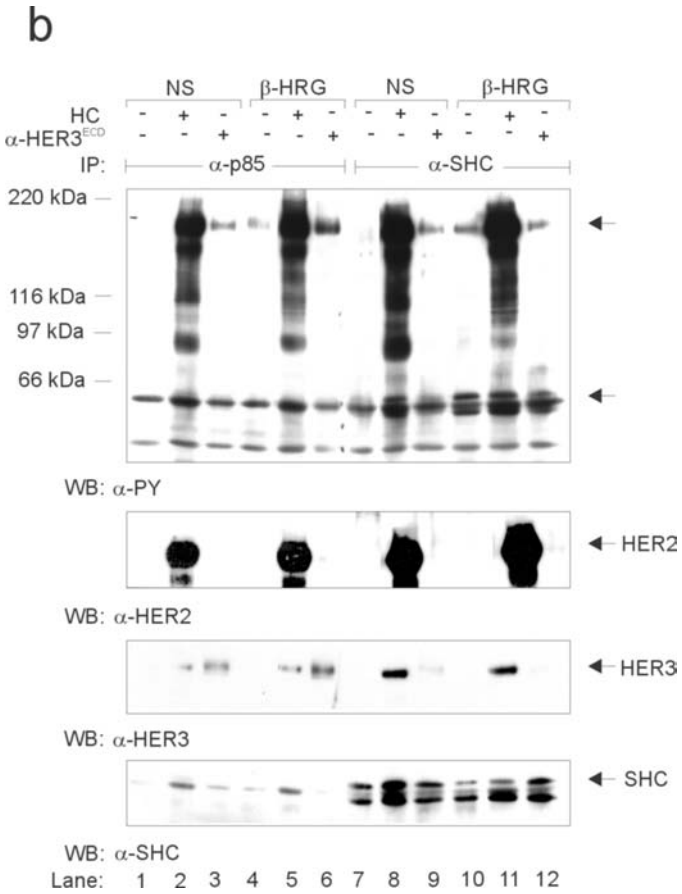

Fig. 17b: **α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of SHC and PI$_3$-K with ErbB3/HER3 in MDA-MB-468 cells.** Serum starved breast cancer cells MDA-MB-468 were pretreated and stimulated as described in figure 16. Cell lysates were either subjected to immunoprecipitation (IP) using monoclonal anti-SHC2 (α-SHC2) or monoclonal anti-PI$_3$-K (α-PI$_3$-K) antibodies. Tyrosine phosphorylation levels were analyzed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-4G10) (**b**, upper panels). Reblots with α-SHC and α- PI$_3$-K antibodies were performed as a control for protein loading (**b**, bottom panels). Coprecipitation of ErbB2/HER2 and ErbB3/HER3 with SHC or with PI$_3$-K was detected by probing the membrane either with α-ErbB2/HER2 or α- ErbB3/HER3 antibody (**b**, middle panes). Unstimulated cells are indicated by NS. Coprecipitating proteins are indicated by an arrow.

As shown in figure 17, α-HER3^{ECD} significantly decreased the tyrosine phosphorylation of SHC (lower arrow) after HRG stimulation in the cell lines MCF-7^{ADR} and MDA-MB-468 (Figure 17a, b compare lane 10 with 12). Interestingly, the association of SHC with the tyrosine-phosphorylated receptors (upper arrow) was strongly inhibited in MCF-7^{ADR} cells but not in MDA-MB-468. The immunoprecipitates of the regulatory subunit of PI_3-K yielded similar results. The binding of tyrosine-phosphorylated ErbB3/HER3 to p85 was decreased in MCF-7^{ADR}, while, interestingly, an increase was observed in MDA-MB-468 (Fig. 17a, b, lanes 4 and 6). HC enhanced binding of the effector proteins SHC and PI_3-K in both cell lines (lane 5). These data show that α-HER3^{ECD} interferes with SHC function by inhibiting its tyrosine phosphorylation. The association of SHC and PI_3-K with ErbB3/HER3 is substantially modified by α-HER3^{ECD} with respect to control treatment in both cell lines: in MCF-7^{ADR}, where ErbB3/HER3 is more abundant than ErbB2/HER2, the association with the effectors is decreased while in MDA-MB-468, where ErbB3/HER3 is present in a lower relative amount than ErbB2/HER2, α-HER3^{ECD} promotes the association of SHC and PI_3-K both under resting and stimulated conditions.

5.9. α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of GRB2 with ErbB2/HER2

Since SHC associates with the adaptor molecule GRB2 after HRG stimulation, we explored the effect of the reduced phosphorylation of SHC in MCF7 cells by measuring GRB2 binding (Fig. 18). Therefore we performed GST-pulldown assays in cells using GST-GRB2 fusions and the same experimental design as before.

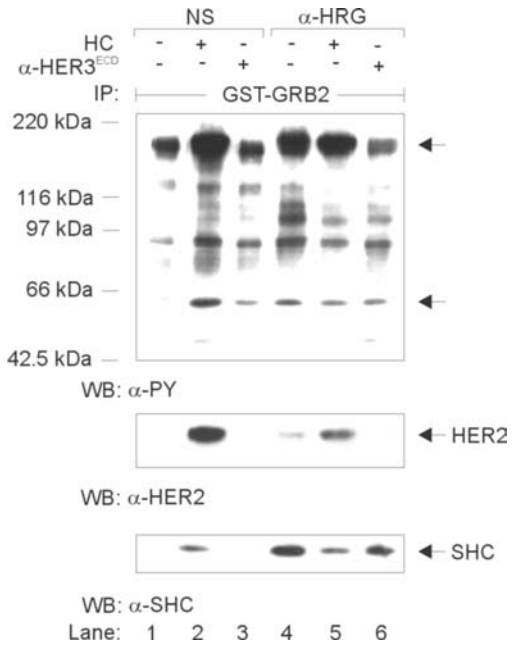

Fig. 18: **α-HER3^{ECD} decreases the phosphorylation of SHC and modifies the association of GRB2 with ErbB2/HER2.** Serum starved breast cancer cells MCF-7^{ADR} were pretreated and stimulated as described in figure 16. Cell lysates were subjected to a GST-GRB2 pulldown assay with 5μg recombinant GST-GRB2 protein coupled to Glutathione Sepharose. Tyrosine phosphorylation levels were analyzed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-4G10) (upper panels). Coprecipitation of ErbB2/HER2 and SHC with GST-GRB2 was detected by probing the membrane either with α-ErbB2/HER2 or α-SHC antibody (middle panes). Unstimulated cells are indicated by NS. Coprecipitating proteins are indicated by an arrow.

Indeed, the reduced tyrosine phosphorylation of SHC observed in MCF-7^{ADR} resulted in a decreased binding of GRB2 to SHC (Fig. 18 bottom panel, compare lane 4 and 6), and a complete inhibition of its association with ErbB2/HER2 (Fig. 18 middle panel).

5.10. α-HER3^{ECD} inhibits JNK1 and PI3-K activity

Given the qualitatively different signalling interference caused by α-HER3^{ECD} in the two cell lines, we asked whether the downstream effects of the antibody would also be different. The adaptor protein SHC regulates MAPK signalling pathways downstream of growth-factor receptors, activating JNK and ERK-2, respectively (Hashimoto et al., 1999; Vijapurkar et al., 1998). To investigate the effect of α-HER3^{ECD} on MAP kinase signalling we performed kinase assays under the same experimental conditions in MCF-7^{ADR} and MDA-MB-468 (Fig. 19).

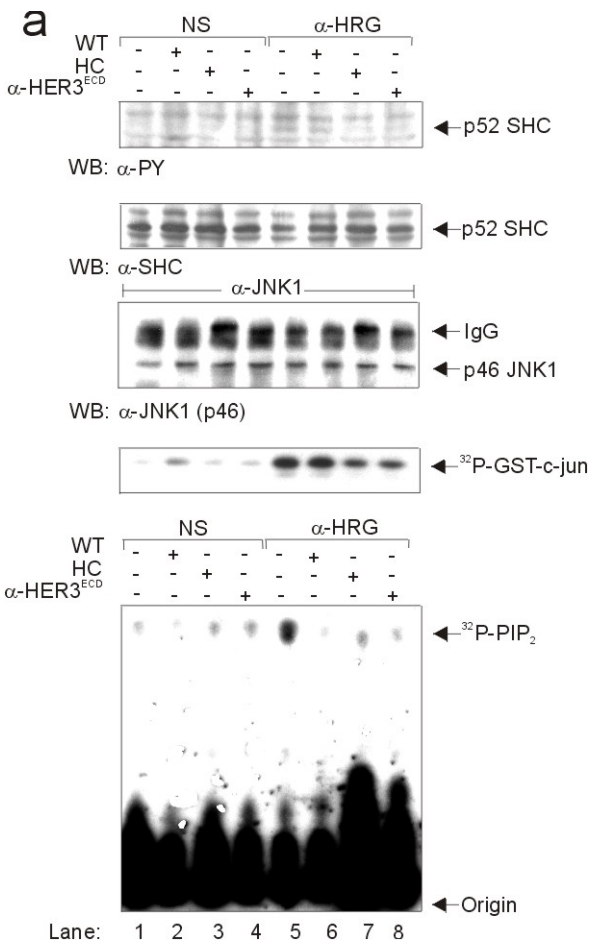

Fig. 19a: **α-HER3^{ECD} inhibits JNK1 and PI3-K activity in MCF-7^{ADR} cells. a**, Serum starved breast cancer cells MCF-7^{ADR} were pretreated either with 50 μg/ml HC or 10 μg/ml α-HER3^{ECD} for 60 minutes or with 100 nM/ml Wortmannin (WT) for 30 minutes and stimulated as described in figure 16. Cell lysates were either used for immunoprecipitation with polyclonal anti-JNK-1 (α-JNK-1) antibody or monoclonal α-4G10 and PI_3-K assays were performed (see Methods section). Whole cell lysates (WCL) were additionally probed with α-PY antibody to confirm effect of α-HER3^{ECD} on SHC tyrosine phosphorylation and after stripping reprobed with α-SHC antibody to determine equal loading of proteins (**a**, middle and upper lower panels). α-JNK-1 immunocomplexes were subjected to JNK-kinase assays (**a**, upper panels). Phosphorylated c-jun is indicated by an arrow. An anti-JNK-1 antibody (α-JNK-1) was used to confirm equal protein loading. PI_3-K activity is strongly diminished after α-HER3^{ECD} pretreatment, due to the inhibition of PI_3-K binding to ErbB3/HER3 (**a**, lower panels). Phosphorylated Phosphatidylinositol is indicated by an arrow.

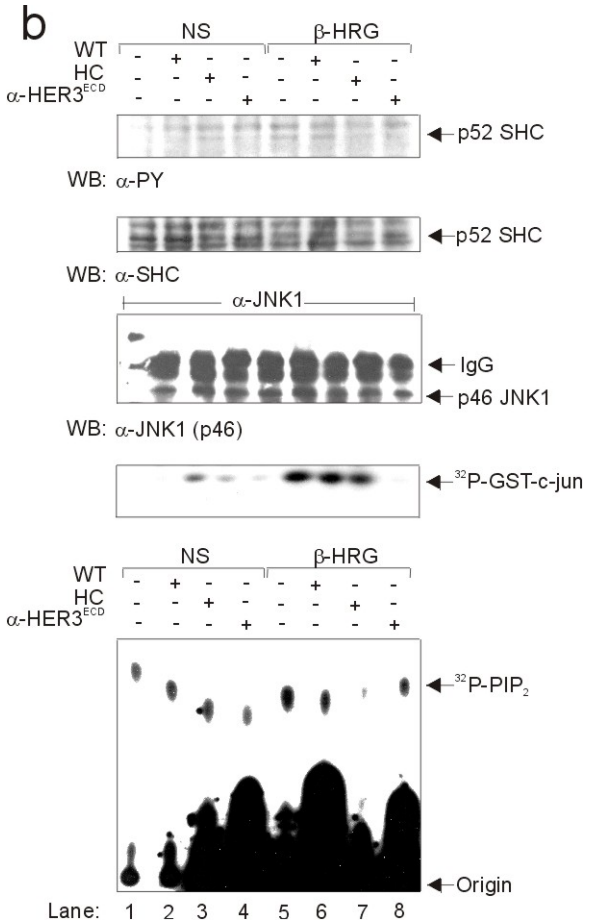

Fig. 19b: **α-HER3^{ECD} inhibits JNK1 and PI3-K activity.** Serum starved breast cancer cells MDA-MB-468 were pretreated either with 50 μg/ml HC or 10 μg/ml α-HER3^{ECD} for 60 minutes or with 100 nM/ml Wortmannin (WT) for 30 minutes and stimulated as described in figure 16. Cell lysates were either used for immunoprecipitation with polyclonal anti-JNK-1 (α-JNK-1) antibody or monoclonal α-4G10 and PI_3-K assays were performed (see Methods section). Whole cell lysates (WCL) were additionally probed with α-PY antibody to confirm effect of α-HER3^{ECD} on SHC tyrosine phosphorylation and after stripping reprobed with α-SHC antibody to determine equal loading of proteins (**b**, middle and upper lower panels). α-JNK-1 immunocomplexes were subjected to JNK-kinase assays (**b**, upper panels). Phosphorylated c-jun is indicated by an arrow. An anti-JNK-1 antibody (α-JNK-1) was used to confirm equal protein loading. PI_3-K activity is strongly diminished after α-HER3^{ECD} pretreatment, due to the inhibition of PI_3-K binding to ErbB3/HER3 (**b**, lower panels). Phosphorylated Phosphatidylinositol is indicated by an arrow.

The pretreatment with α-HER3^{ECD} determined a strong decrease of JNK activity in both cell lines (Fig.19a, b, compare lane 5 and 8); strikingly, the inhibitory effect of α-HER3^{ECD} on JNK kinase activity was even stronger in MDA-MB-468 than in MCF-7^{ADR}. HC, on the other hand, only reduced JNK activity in MCF-7^{ADR} (Fig. 19a). ERK-2 activity was significantly decreased after pretreatment with α-HER3^{ECD}, whereas HC had no effect (data not shown). Since an involvement of PI_3-K in carcinoma invasion has recently been demonstrated (Shaw et al., 1997), we investigated the inhibitory properties of α-HER3^{ECD} on PI_3-K activity (Fig. 19a, b, bottom panels). In both cell lines α-HER3^{ECD} resulted in a strong decrease of PI_3-K activity upon HRG stimulation (Fig. 19a, b). In MDA-MB-468 HC exerted an even greater inhibitory effect on PI_3-K activity than α-HER3^{ECD}, which could be due to the threefold higher expression of ErbB2/HER2 than in MCF-7^{ADR}. Wortmannin (WT), a widely used inhibitor of PI3K, was also used as a control pretreatment (lanes 2,6). Taken together, these results have a potential therapeutic significance, because they show that α-HER3^{ECD} differentially modifies early signalling events (i.e. receptor phosphorylation, PI_3-K and SHC association) according to the ErbB2/HER2:ErbB3/HER3

ratio, but invariably decreases the activity of downstream effectors regardless of the ErbB2/HER2:ErbB3/HER3 ratio.

5.11. Effect of α-HER3^{ECD} and Herceptin® on the endocytosis of ErbB3/HER3 and ErbB2/HER2 after HRG stimulation

We then tried to dissect the molecular mechanisms leading to α-HER3^{ECD}-induced inhibition of ErbB3/HER3 tyrosine phosphorylation. It is known that ErbB2/HER2 and ErbB3/HER3 are endocytosed and recycled after HRG stimulation (Yarden and Sliwkowski, 2001). To assess whether α-HER3^{ECD} interferes with receptor recycling, we determined the surface expression of ErbB3/HER3 after incubation with the antibody for different time periods and subsequent stimulation with HRG for 5 minutes. ErbB3/HER3 was immunoprecipitated after biotinylation of membrane proteins and the corresponding phosphotyrosine content assessed by western blot in whole cell lysates (Fig. 20).

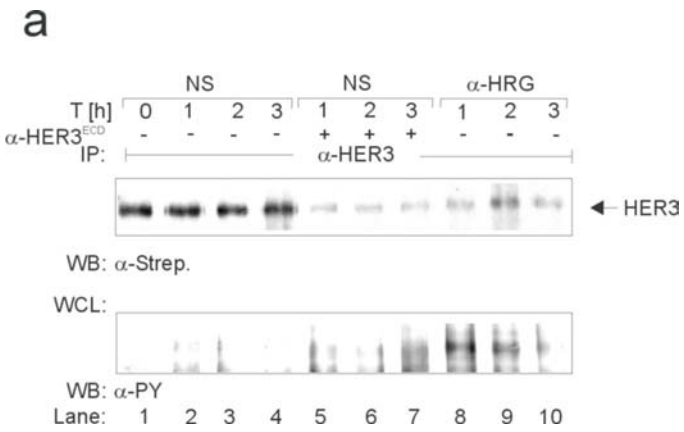

Fig. 20a: **Effect of α-HER3^{ECD} and HRG on the endocytosis of ErbB3/HER3.** Serum starved MCF-7^{ADR} cells were incubated at 4°C with 10 μg/ml α-HER3^{ECD} or with 5 μg ml^{-1} α-Heregulin (HRG) for 1 h, then washed two times with PBS and incubated at 37°C for the indicated timepoints. At the end of incubation, cells were biotinylated and lysed (see Method section). Cell lysates were subjected to immunoprecipitation (IP) using monoclonal monoclonal anti-ErbB3/HER3 (α-ErbB3/HER3) antibodies (**a**, upper panel). Tyrosine phosphorylation level of whole cell lysates (WCL) was analyzed by western

blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-4G10) (**a**, lower panels). NS, unstimulated cells.

As shown in Fig. 20a (upper panel), α-HER3^{ECD} caused a long-lasting decrease in the surface expression of ErbB3/HER3; the activation state of the receptor after HRG stimulation was, at all pretreatment time points, indistinguishable from that of control untreated cells (Fig. 20a, lower panel). The treatment with HC had strikingly different effects on ErbB2/HER2 recycling, resulting in a strong stabilisation of ErbB2/HER2 at the cell surface and a high level of tyrosine phosphorylation (Fig. 20b and 16a). For comparison, we treated the cells for equivalent time periods with the physiological ligand, HRG.

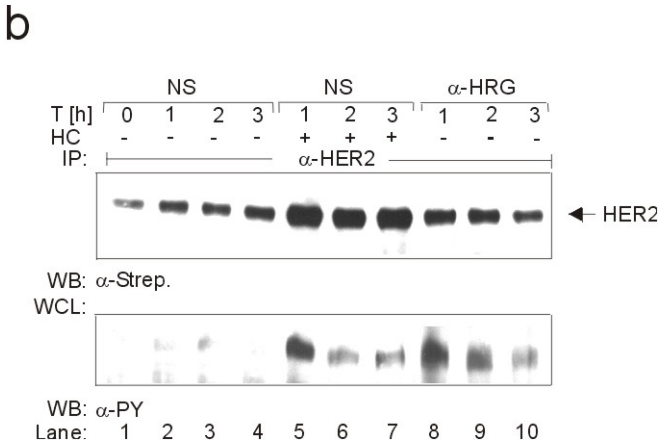

Figure 20b: **Effect of Herceptin® and HRG on the endocytosis of ErbB2/HER2.** Serum starved MCF-7^{ADR} cells were incubated at 4°C with 50 μg/ml HC or with 5 μg ml^{-1} α-Heregulin (HRG) for 1 h, then washed two times with PBS and incubated at 37°C for the indicated timepoints. At the end of incubation, cells were biotinylated and lysed (see Method section). Cell lysates were subjected to immunoprecipitation (IP) using monoclonal anti-ErbB2/HER2 (α-ErbB2/HER2) antibodies (**b**, upper panel). Tyrosine phosphorylation level of whole cell lysates (WCL) was analyzed by western blotting (WB) with monoclonal anti-phosphotyrosine antibody (α-4G10) (**b**, lower panels). NS, unstimulated cells.

This also determined the downregulation of ErbB3/HER3 from the cell surface (although a relative increase in surface-ErbB3/HER3 was reproducibly observed after 2hs incubation, figure 20a lane 9), but the receptor, as expected, was still highly phosphorylated after two hours of incubation with the ligand. The effects of HRG pretreatment on ErbB2/HER2 downregulation were modest and a significant fraction of the receptor molecules were still on the cell surface after 3 hs of treatment (Fig. 20b, lanes 8-10). Mechanistically, we hypothesize that the strong general inhibitory effects of α-HER3^{ECD} on HRG signalling could be due to increased ErbB3/HER3 endocytosis, which prevents the receptor from forming active signalling complexes with ErbB2/HER2 at the cell surface. The different effects of the two antibodies on the endocytosis rate of ErbB2/HER2 and ErbB3/HER3 could be due to their different epitope binding properties and effectiveness in inducing conformational changes in the receptor.

5.12. The proliferative and migratory properties of breast cancer cell lines are inhibited by α-HER3^{ECD}

We then asked whether the ability of α-HER3^{ECD} to attenuate HRG-mediate activatory signals would also result in a functional output, i.e. the inhibition of growth and invasiveness of breast cancer cells. In order to assess the effect of α-HER3^{ECD} on the migratory and proliferative properties of breast cancer cells, we performed BrdU-incorporation assays in the presence or absence of α-HER3^{ECD} and stimulated with HRG. Pretreatment with α-HER3^{ECD} decreased proliferation by 28.7% +/- 6.18% and 21.1% +/- 7.62% in MCF-7^{ADR} and MDA-MB-468 respectively. HC, on the other hand, had no effect in these cell lines (data not shown) (Fig. 21a).

a

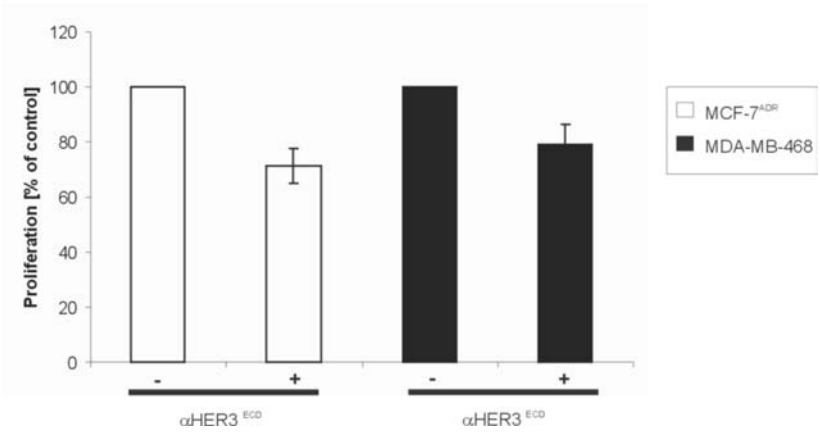

Fig. 21a: **α-HER3^{ECD} inhibits the HRG-induced proliferation of breast cancer cell lines.** MCF-7^{ADR} and MDA-MB-468 were pretreated with α-HER3^{ECD} for 1 hr and subsequently stimulated with HRG for 16 hr. BrdU incorporation was carried out as described in the Methods section. The results are the average of triplicate samples from at least four independent experiments. All data are expressed as means +/- standard deviation represented as error bars. Comparisons were made using Student's t-test with $P<0.05$ being considered statistically significant.

To investigate the effect of α-HER3^{ECD} on the migratory properties of breast cancer cells, we conducted chemotaxis experiments with MCF-7^{ADR} and MDA-MB-468 in the presence or absence of α-HER3^{ECD}. We observed a strong decrease in migration of 59.1% (*P*=0.018) and 55.4% (*P*=0.00005) in MCF-7^{ADR} and MDA-MB-468, respectively (Fig. 21b). Our data thus clearly show an inhibitory effect of α-HER3^{ECD} on proliferation and migration in MCF-7^{ADR} and MDA-MB-468.

b

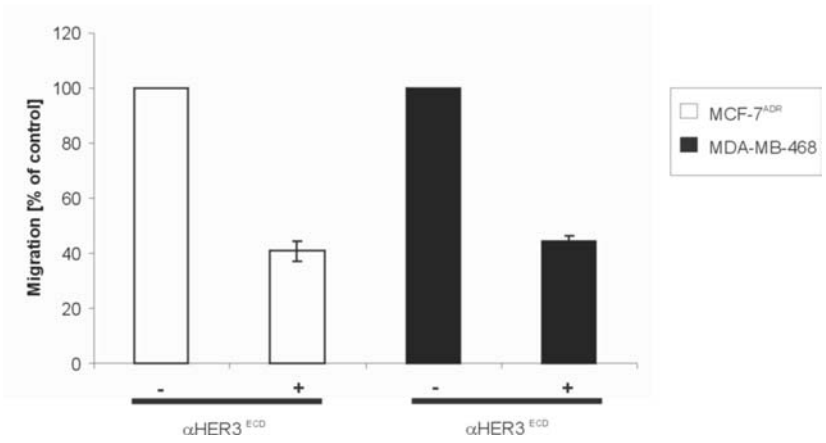

Fig. 21b: **α-HER3^{ECD} reduces the migratory response of breast cancer cell lines to chemoattractants.** The cells were pretreated with α-HER3^{ECD} for 1 hr, detached and transferred to the upper well of a Boyden Chamber. The assay was carried out in the presence of the chemoattractant for 16 hr as described in the Methods section. The migrating cells present on the lower side of the membrane were stained and counted. The results are the average of triplicate samples from two independent experiments. Statistical analysis was performed as described in figure 21a.

6. Discussion

6.1. The role of ErbB3/HER3 in glioma invasion

The cytoplasmic protein tyrosine kinase PYK2 lies at the convergence point of pathways that transmit signals from stimulated integrins, G protein-coupled receptors and RTKs to downstream effectors (Avraham et al., 2000; Dikic et al., 1996). An important stimulus that activates PYK2 is α-HRG (Zrihan-Licht et al., 2000). Both PYK2 and HRG are predominantly expressed in the central nervous system, and the genes encoding the two proteins are localised in close proximity to each other on chromosome 8 (Inazawa et al., 1996). HRG is a promiscuous ligand for HER3 and ErbB4/HER4, members of the EGFR family of RTKs, which are among the most potent regulators of cell proliferation, differentiation and motility. In glioblastoma cell lines, which are devoid of ErbB4/HER4, binding of HRG leads to formation of ErbB2/HER2-ErbB3/HER3 heterodimers allowing transphosphorylation of ErbB3/HER3 by ErbB2/HER2 (Klapper et al., 2000). The signalling molecules SHC and PI_3-K are known to bind to the C-terminal region of HER3 and to promote cell growth (Klapper et al., 2000; Wallasch et al., 1995; Yarden and Sliwkowski, 2001). These pieces of information, obtained in several model systems, prompted us to explore an HRG-stimulated signalling pathway involving ErbB2/HER2/HER3 and PYK2 in glioblastoma cell lines. Based on the data presented in this thesis, we propose a model in which PYK2 is phosphorylated by ErbB3/HER3 upon HRG stimulation, and induces cell motility and invasiveness through the MAPK pathway (Fig. 22).

6.1.1. PYK2 associates with ErbB3/HER3

Immunoprecipitation assays indicate a constitutive association of PYK2 with ErbB3/HER3, which is promoted by ErbB2/HER2 activity (Fig. 8b). This finding is supported by immunofluorescense studies, which confirmed a constitutive colocalization of these signalling molecules in a punctuated pattern throughout the cytoplasm independent of HRG stimulation (Fig 9). It is known that ErbB3/HER3 is internalised through the clathrin-mediated endocytotic pathway (Waterman and Yarden, 2001) and similar punctuated distributions have recently been shown for several proteins associated with this pathway, including mHip1r and EGFR (Engqvist-Goldstein et al., 1999; Sorkina et al., 1999). Centripetal movement of the clathrin-coated vesicles towards the perinuclear region, which occurs on a time scale of several minutes, has been directly demonstrated by using a GFP-chlathrin fusion protein in *Dictyostelium* and COS-1 cells (Damer and O'Halloran, 2000; Gaidarov et al., 1999). A prolonged activation state of ErbB3/HER3/PYK2 complexes within endosomes during recycling would enable recurrent association of other signalling molecules and thus serve to amplify the initiating signal. This prolonged accessibility of ErbB3/HER3/PYK2 complexes and their transport towards the site of MAPK activity could explain the exceptionally strong mitogenic potential of ErbB2/HER2-ErbB3/HER3 heterodimers, compared to other members of the EGFR family (Klapper et al., 2000). Indeed, it has been shown that ErbB2/HER2-ErbB3/HER3 heterodimers are getting recycled, whereas EGFR-containing dimers are degraded via the ubiquitination pathway (Waterman and Yarden, 2001).

6.1.2. HRG stimulates PYK2 activation

Although association of PYK2 with ErbB3/HER3 and its recycling appear to be HRG-independent, tyrosine phosphorylation of PYK2 is elevated by HRG stimulation. Our evaluation of the phosphorylation mechanism indicates that the kinase activity of ErbB3/HER3 is critical for this process (Fig. 10). Specifically, tyrosine residues Y1257, Y1270 and Y1288 in the C-terminal region of ErbB3/HER3 are important for PYK2 transphosphorylation and its physical association with HER3. Furthermore, *in vitro* kinase assays substantiate the direct phosphorylation of GST-PYK2-CT by endogenous ErbB3/HER3 and GST-HER3-KD (Fig. 10 and 11). In selective inhibition experiments, we excluded ErbB2/HER2, src and PI_3-K as proteins that directly phosphorylate PYK2.

6.1.3. PYK2 is necessary for MAPK activation

In addition, the dominant-negative PYK2-KM abrogates MAPK activation, suggesting that mitogenicity correlates with PYK2 activity (Fig. 13). It has been shown that cells overexpressing PYK2 exhibit elevated tyrosyl-phosphorylated SHC and subsequent ERK-2 activity (Lev et al., 1995). We did not observe a direct interaction between PYK2 and SHC (data not shown), but it has been proposed recently that SHC associates with PYK2 through GRB2 in platelets in dependence of αIIbβ3 integrin activation, thus linking extracellular signals to the Ras/Raf pathway (Ohmori et al., 2000). GRB2-binding to activated PYK2 could explain the subsequent tyrosine phosphorylation of SHC, which contributes to increased cellular downstream responses. Interestingly, PYK2-KM was also found to attenuate PI_3-K activity (Fig. 14). ErbB3/HER3 harbours six potential docking sites for the SH2 domain of the PI_3-K subunit p85, and one proline-rich sequence that forms a consensus binding site for the SH3 domain of p85, all potentially contributing to a strong association of p85 with ErbB3/HER3 (Hellyer et al., 1998). Furthermore, a constitutive association between PYK2 and p85 in platelets was reported, where a YXXM motif in PYK2 could serve for binding to the SH2 domain of p85

(Sayed et al., 2000). Indeed, immunoprecipitation of p85 from cell lysates revealed HRG-dependent association of tyrosyl-phosphorylated proteins of M_r= 113 kDa and 180 kDa, which were identified as PYK2 and ErbB3/HER3 (data not shown), suggesting that ErbB3/HER3, PYK2 and PI_3-K are components of a multiprotein signalling-complex.

6.1.4. ErbB3/HER3 is a kinase with narrow substrate specificity

Previous studies analyzing ErbB3/HER3 have shown that its kinase activity is impaired due to the presence of non-consensus residues at positions 740 and 815 compared to all other tyrosine kinases, but made use of the artificial substrates poly($Glu^{80}Tyr^{20}$) or Mn•TNP-ATP, which appear to be inadequate for ErbB3/HER3 (Guy et al., 1994; Sierke et al., 1997). Additionally, ErbB3/HER3 kinase activity could not be detected in the myeloid cell line 32D, devoid of any ErbB proteins, which has been extensively used by many groups as a tool for investigating the ErbB2/HER2-ErbB3/HER3 signalling pathway (Yarden and Sliwkowski, 2001). This result however could be explained by the fact, that ErbB3/HER3 is a kinase with narrow substrate specificity and that the substrate PYK2 is not expressed in 32D cells. On the other hand, structural and biochemical data are compatible with the ability of ErbB3/HER3 to phosphorylate PYK2, since ErbB3/HER3 can bind ATP and apparently possesses a functional kinase, although its activity is weak in comparison to the other EGFR family receptors (Guy et al., 1994; Sierke et al., 1997). In fact, a biochemical study clearly demonstrated that ErbB3/HER3 has an active kinase, which however is of two orders of magnitudes lower than the EGFR kinase activity (Guy et al., 1994). Furthermore, various groups have reported effects controlled by ErbB3/HER3 activation in the breast cancer cell lines BT483 and T47D, in the breast epithelial cell line MCF-10A and in the ovarian cancer cell line OVCAR-3, apparently independent of the contribution of other EGFR family members (Beerli et al., 1995; Rajkumar and Gullick, 1994). Although they hypothesised that ErbB3/HER3 could be the kinase

controlling the observed phosphorylation event, the authors favored the hypothesis that an as yet unidentified cytoplasmic kinase or another ErbB family member would be directly responsible for this effect (Beerli et al., 1995). Clearly, the existence of additional EGFR family members that associate and cooperate with ErbB3/HER3 has not been revealed by sequence analysis of the human genome, which renders this possibility invalid.

In a mechanistic model we propose that PYK2 is a key element in transmitting HRG-induced signals. Part of the signalling from PYK2 to ERK-2 is transmitted through PI_3-K and SHC, but is also to some extent independent of ErbB2/HER2 (Fig. 13 and 14). These findings suggest that PYK2 is involved in the control of multiple downstream effectors, which modulate MAPK pathways (Fig. 22).

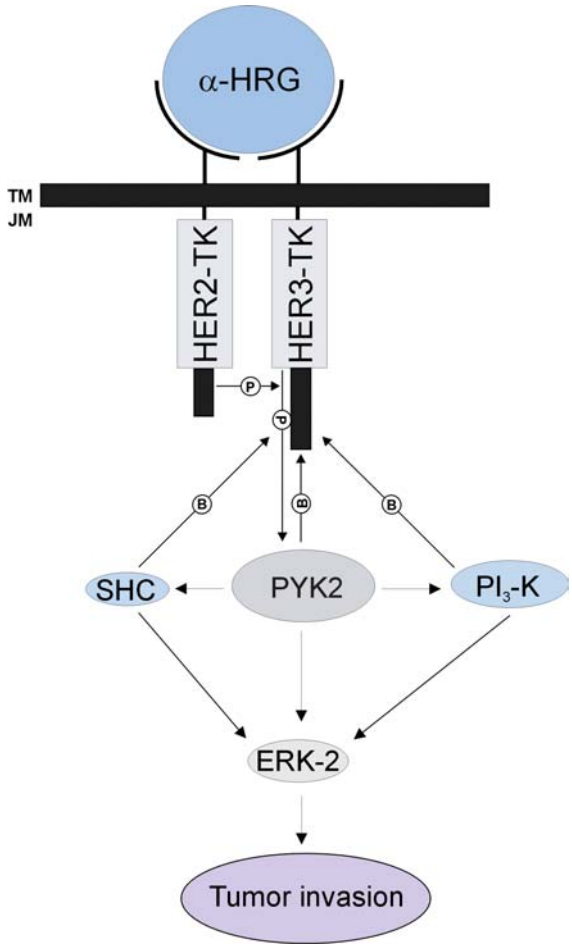

Figure 22: **Role of PYK2 in ErbB2/HER2-ErbB3/HER3 signalling.** The model indicates a novel signal transduction pathway, which leads from α-HRG stimulation to MAPK activation and induces tumour invasion. For details, see discussion. TM indicates the transmembrane domain, JM the juxtamembrane region. Arrows with an encircled B or P indicate binding and phosphorylation, respectively.

6.1.5. Dominant-negative PYK2 suppresses tumourinvasion

Finally, we show that the dominant-negative mutant PYK2-KM suppresses tumour invasiveness in three glioma cell lines (Fig. 15), which correlates with an inhibition of ERK-2 activity (Fig. 14). Interestingly, PYK2-KM abrogated invasiveness to the same extent as inhibition of MEK1 with PD98059, which strongly suggests that PYK2 via the MAPK pathway plays a key role in glioma invasion. It has been shown that ERK activity can regulate myosin phosphorylation, leading to actin-myosin association and cell contraction of the extracellular matrix, and that ERK can facilitate cell invasion and protect cells from apoptosis (Brunet et al., 1999; Nguyen et al., 1999). Concurrent with these findings, increased MAPK activity in our cell systems induced an increased invasive behaviour of glioma cells. Taken together, our data show that ErbB3/HER3 does not only cooperate with other EGFR family members by heterodimer formation, but that it also is an active kinase with an apparently very selective substrate specificity. In glioma cells, PYK2 is directly phosphorylated by ErbB3/HER3, which results in a potentiated PI_3-K activity and enhanced signal through ERK-2, and a strongly invasive behaviour of the tumour cells. Our findings establish a new signalling pathway involving a functional connection between ErbB3/HER3 and PYK2 and provide a new basis for the development of therapies against invasive glioma.

6.2. ErbB3/HER3 as a target in breast cancer therapy

ErbB2/HER2-ErbB3/HER3 heterodimers are fundamental regulators of breast cancer cell growth. Clinical studies have shown that the overexpression of ErbB2/HER2 and ErbB3/HER3 is associated with the lack of response to hormonal therapy, high malignancy and poor prognosis. The most highly metastatic breast cancer cell lines, on the other hand, lack the estrogen receptor and express endogenously HRG, which promotes their growth through an autocrine loop. Our results indicate that an antibody directed against the extracellular domain of ErbB3/HER3 efficiently decreases the tyrosine-phosphorylation of ErbB2/HER2 after HRG stimulation in the breast cancer cell lines MCF-7^{ADR} and MDA-MB-468; this inhibits the activation of downstream effectors and ultimately limits the proliferation and motility properties of the cells.

6.2.1. α-HER3^{ECD} promotes ErbB3/HER3 internalisation and inhibits tyrosine phosphorylation of ErbB2/HER2

α-HER3^{ECD} accelerates the endocytosis of ErbB3/HER3, thus resulting in the clearance of ErbB3/HER3 from the cell surface and potentially preventing HRG-mediated ErbB2/HER2-ErbB3/HER3 heterodimerisation. The inhibitory effects of α-HER3^{ECD} are strikingly potent; this antibody counteracts the activity of the ErbB2/HER2-ErbB3/HER3 complex, the most active signal transducer among the EGFR family members, characterized by a slow endocytosis rate and by the ability of signalling throughout the internalization process.

The action mechanism of the therapeutical anti-ErbB2/HER2 antibody Herceptin® in our cell system is substantially different, since Herceptin® induces hyperphosphorylation of ErbB2/HER2 and it does not abrogate the transphosphorylation activity of ErbB3/HER3 by ErbB2/HER2 after HRG stimulation. Herceptin® has been reported to sensitize breast cancer cells to TNF-alpha, Trance and various chemotherapeutics and to induce cell cycle arrest due to p27kip expression (Cuello et al., 2001; Lane et al.,

2001; Sliwkowski et al., 1999). Still, these effects are not sufficient to provide a tumour clearance in living organisms. A recent study by Clynes *et al.* has shown that the primary effect of Herceptin® is to stimulate the immune system by binding to the FcγR receptor (Clynes et al., 2000). They have elegantly demonstrated by gene targeting in mice that an antibody endowed with the ability to bind FcγR receptor, but not the FcγRIIB subtype, results in the complete tumour eradication from the organism. However, as extensively discussed by Clynes *et al.*, the inhibition of the growth and invasion promoting signals within the tumour cell is predicted to increase the chances of the immune system to efficiently fight the malignancy (Clynes et al., 2000). Given that the inhibitory effects of α-HER3^{ECD} on ErbB2/HER2-ErbB3/HER3 signalling are stronger than those of Herceptin®, the former antibody could be an even more effective chemotherapeutic agent, provided that it has an equivalent ability to activate the effector cells of the immune system.

6.2.2. α-HER3^{ECD} inhibits JNK signalling

An important feature of α-HER3^{ECD} is its ability to completely block JNK activity (Fig. 19). JNK is involved in the control of cell migration (Xia et al., 2000) and several lines of evidence have also recently demonstrated the specific importance of JNK signalling in breast cancer cell motility and growth. Very invasive breast cancer cell lines were shown by microarray analysis to overexpress JNK (Zajchowski et al., 2001). Additionally, it has been shown that the prolylisomerase PIN1, overexpressed in breast cancer, cooperates with Ras in enhancing JNK activity; this, in turn, controls cyclin D1 promoter and increases the transcription of cyclin D1 (Wulf et al., 2001). CyclinD1 is overexpressed in more than 50% of diagnosed breast carcinomas and has been shown to have a causative role in the pathogenesis of breast cancer induced by *neu*, the oncogenic variant of ErbB2/HER2 (Yu et al., 2001). α-HER3^{ECD} abrogates JNK activity more efficiently than Herceptin® by interfering with SHC binding to

the C-terminal domain of ErbB3/HER3. Both α-HER3^{ECD} and Herceptin®, on the other hand, efficiently inhibit PI3K activity, another important determinant of proliferation and invasiveness of breast cancer cells.

Although the cell lines used in this study are characterized by a different ErbB2/HER2-ErbB3/HER3 ratio, namely 1:3 for MCF7-ADR and 3:1 for MDA-MB468. α-HER3^{ECD} could effectively inhibit receptor tyrosine phosphorylation, proliferation and migration in both cases. This finding is of potential significance for the perspective therapeutical use of anti-ErbB3/HER3 antibodies, because beneficial effects could be expected in a wide spectrum of clinical cases presenting different relative amounts of ErbB2/HER2 and ErbB3/HER3.

Taken together, our data show that ErbB3/HER3, in association with ErbB2/HER2, plays a fundamental role in the control of breast cancer cell growth and invasiveness. Moreover, ErbB3/HER3 emerges for the first time as an important determinant of the breast cancer cell behavior as well as a target for drug design. Our findings provide a strong biological basis for the idea that counteracting aberrant ErbB3/HER3 signalling could be a primary objective in antibody-based breast cancer therapy.

7. Abbreviations

α	anti-
ADCC	Antibody-dependent cellular cytotoxicity
ATCC	American type culture collection
ATP	Adenosintriphosphate
BBS	2 x BES buffered Saline
BES	N-, N-Bis(Hydroxyethyl)-2-Aminoethane-Sulfoneacid
bp	Basepair
CDC	complement-dependent cytotoxicity
cDNA	complementary DNA
Ci	Curie
DKFZ	Deutsches Krebsforschungs-Zentrum
DMSO	Dimethylsulfoxid
DNA	Desoxyribonukleicacid
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	Ethylendiaminetetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK-2	Extracellular-signal regulated kinase 2
FCS	Fetal Calfserum
Fig.	Figure
g	Gramm
gp	glycoprotein
GST	Glutathione-S-Transferase
GRB 2	Growth factor bound 2
h	hour
HC	Herceptin®
HEPES	N-(2-Hydroxyethyl)-piperazine N'-2-ethanesulfoneacid
HER2, 3, 4	human EGF-Receptor 2, 3, 4
HRG	Heregulin
IP	Immunoprecipitation
kDa	kiloDalton
m	meter
M	Molar
mAB	monoclonal antibody

MAPK	Mitogen-activated protein kinase
min	minute
ml	Milliliter
NDF	*Neu* Differentiation Factor
NRG	Neuregulin
PAGE	Polyacrylamide-Gelelectrophorese
pAB	polyclonal antibody
PBS	Phosphate-buffered Saline
PCR	Polymerase chain reaction
PI_3-	Phosphatidyl-inositol-3-OH
PMSF	Phenyl-Methyl-Sulfonyl-Fluoride
PY	Phosphotyrosine
PYK2	Proline-rich tyrosine kinase 2
rpm	Rounds per minute
RT	Roomtemperature
RTK	Receptortyrosinekinase
s	Second
SDS	Sodiumdodecylsulfate
Tab.	Table
TGF	Transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
O/N	over night
UV	Ultraviolet light
V	Volt
VSV	vesicular somatitis virus glycoprotein
Vol.	Volume
WM	Wortmannin
WT	wildtype

8. Literature

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PUBLICATIONS/PATENTS

Htun van der Horst, E., Weber, I., and Ullrich. A. PYK2 phosphorylation by HER3 mediates Heregulin-induced glioma invasion (submitted).
Htun van der Horst, E., Murgia, M., and Ullrich. A. Monoclonal HER3 antibodies in breast cancer therapy (manuscript in preparation).

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