# Intracellular trafficking of FGF1 endocytosed by its four tyrosine kinase receptors

**Ellen Margrethe Haugsten**

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**DEPARTMENT OF BIOCHEMISTRY,**
**INSTITUTE FOR CANCER RESEARCH,**
**THE NORWEGIAN RADIUM HOSPITAL**

**DEPARTMENT OF MOLECULAR BIOSCIENCES,**
**UNIVERSITY OF OSLO,**
**NORWAY**

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# 1. Acknowledgment

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Ellen Margrethe Haugsten

# 2. Abbreviations

| | |
|---|---|
| Akt | Protein kinase B |
| ATP | Adenosine triphosphate |
| CFR | Cysteine rich FGF receptor |
| D1/D2/D3 | Immunoglobulin like domain 1/2/3 |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ER | Endoplasmic reticulum |
| ERC | Endocytic recycling compartment |
| ERK | Extracellular-signal-regulated kinase |
| ESCRT | Endosomal sorting complex required for transport |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| FRS2 | FGF receptor substrate 2 |
| Hrs | Hepatocyte-growth-factor-regulated tyrosine kinase substrate |
| HSPG | Heparan sulfate proteoglycan |
| KGF | Keratinocyte growth factor |
| KGFR | Keratinocyte growth factor receptor |
| LDL | Low-density lipoprotein |
| MAPK | Mitogen-activated protein kinase |
| MHC | Major histocompatibility complex |
| MVB | Multivesicular body |
| NGF | Nerve growth factor |
| NLS | Nuclear localization signal |
| PDGF | Platelet-derived growth factor |
| PKC | Protein kinase C |
| PLC | Phospholipase C |
| PTB | Phosphotyrosine-binding domain |
| RTK | Receptor tyrosine kinase |

| | |
|---|---|
| SH2 | Src homology domain |
| SHP-2 | Src homology 2 tyrosine phosphatase |

# 3. Aims of the present study

Fibroblast growth factors and the four related high-affinity, tyrosine kinase fibroblast growth factor receptors are involved in the regulation of many key cellular responses in developmental and physiological processes. Irregularities in FGF-mediated signalling are implicated in several serious disorders such as cancer and various forms of dwarfism. Little is known about the fate of endocytosed fibroblast growth factors and their receptors and the main purpose of this project is to study and compare the intracellular trafficking of the fibroblast growth factor 1 and the four related tyrosine kinase fibroblast growth factor receptors upon internalization.

# 4. Introduction

Uptake of nutrients and communication between cells and their environment occurs through the plasma membrane. Several mechanisms for uptake of nutrients and molecules that are present in the extracellular milieu have evolved in eukaryotic cells. Small molecules such as ions and water enter the cell through plasma membrane proteins that form transporters, channels and pumps. Macromolecules that are too large to enter the cell through these mechanisms are internalized through phagocytosis or pinocytosis. Phagocytosis or cell eating is an active and highly regulated process involving ingestion of large particles such as microorganisms and dead cells by specific cell-surface receptors and signalling cascades[2]. Pinocytosis occurs in all cells and encompasses several mechanisms by which the cell internalizes fluids and solutes from the extracellular milieu into small endocytic vesicles derived from the plasma membrane. The endocytic material is then targeted for several possible fates including fusion with the endosomal compartments followed by degradation or recycling back to the cell surface. The most efficient uptake occurs when solutes are captured by specific high-affinity receptors which then become concentrated into the endocytic transport vesicles[23].

In addition to supplying the cells with nutrients, endocytosis functions to control signalling activity at the cell surface. Cells in the body communicate by secreting chemical signals such as hormones and growth factors, which can be recognized by specific receptors on recipient cells. Binding of a chemical signal to its receptor may induce a variety of different responses in the cell, such as stimulation to divide, migrate or differentiate into a different cell type. Cell-surface receptors are key elements in cellular communication. To avoid overstimulation of the recipient cell, the activated receptors and their corresponding ligands are removed from the cell-surface by endocytosis. Upon endocytosis, the signalling from the activated receptors attenuates as they are degraded in the lysosomes. Defects in receptor-mediated endocytosis including failure to attenuate signalling and impaired delivery of

endocytosed material, are implicated in several diseases such as cancer[55] and hypercholesterolemia[63].

## 4.1 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are transmembrane molecules positioned at the cell surface to detect the presence of corresponding growth factors in the extracellular milieu. The binding of a signal protein to the ligand-binding domain on the outside of the cell results in formation of a dimer ligand-receptor complex that activates the intracellular tyrosine kinase domain of the receptors by autophosphorylation (cross-phosphorylation). Once activated, the kinase domain transfers a phosphate group from ATP to selected tyrosine side chains, both on the receptor proteins themselves and on intracellular signalling proteins. These phosphotyrosines can provide docking sites for downstream effectors containing Src homology (SH2) or phosphotyrosine-binding (PTB) domains and initiates a network of signalling pathways that relay cell-surface signals to the nucleus and other intracellular destinations[121].

These pathways include the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, the phospholipase Cγ (PLCγ)/protein kinase C (PKC) pathway, the phosphatidylinositol 3-kinase pathway and the pathways that regulate small GTPases, such as Rho, Rac and Cdc42[121]. RTKs have also been reported to directly activate signal transducers and activators of transcription (STAT) proteins[27]. The combinatorial information provided by these signal transduction pathways can explain the biological responses of cells to growth factors.

## 4.2 Endocytosis and intracellular sorting of receptor tyrosine kinases

Growth factor-RTK complexes formed at the plasma membrane are not stagnant or restricted to the cell surface. The ligand-receptor complexes can be selectively

recruited into small areas of the plasma membrane that can invaginate inward and pinch off to form vesicles in the cytoplasm. Ligand-RTK complexes are found to be internalized through clathrin-mediated endocytosis[17,44,54], as well as caveolin-mediated endocytosis[38] and clathrin- and caveolin-independent endocytosis[20,21]. Endocytosis by clathrin-dependent and clathrin-independent mechanisms delivers receptors to peripherally located tubular-vesicular structures called sorting or early endosomes (Figure 1).

The sorting endosome is the first main branch point in the receptor-mediated endocytic pathway. Molecules in the sorting endosomes can be sorted to late endosomes, back to the plasma membrane or to the endocytic recycling compartment (ERC) and then back to the surface. The most comprehensive studies of RTK endocytosis have been carried out using the epidermal growth factor (EGF) receptor as an experimental model. Ligand-free EGF receptors are almost exclusively recycled to the cell surface, while ligand-occupied receptors are sorted to lysosomes. However, a small fraction of the occupied EGF receptors can also recycle via the ERC or directly from sorting endosomes back to the cell surface[130].

The targeting of transmembrane proteins to late endosomes/lysosomes from sorting endosomes functions to terminate signalling, as well as to make the cells unresponsive to further signal input until a new complement of receptors has been synthesized. The attachment of ubiquitin to the cytoplasmic part of a membrane protein is thought to function as a signal for lysosomal degradation[51,113]. Ubiquitin is an 8.5 kDa protein that can be covalently linked to lysine residues, and ubiquitination was first described as a mechanism for targeting cytosolic proteins for degradation by the proteasome. Poly monoubiquitination of the EGF receptor has been shown to be necessary for both internalization of the receptor and also for targeting the protein to invaginated membranes in sorting endosomes[51]. Hrs (hepatocyte-growth-factor-regulated tyrosine kinase substrate) links the ubiquitinated receptors to flat clathrin lattices on endosomes[111,112,114] and this interaction might be important for retaining ubiquitinated receptors in maturating endosomes, which would lead to their delivery

to late endosomes. In a second sorting step, the ESCRT (endosomal sorting complex required for transport) protein complexes have been shown to recognize and sort ubiquitinated proteins for delivery to the vacuole lumen[65]. The ESCRT complexes were first identified in yeast, but the emerging picture provides an evolutionary conserved function of the ESCRT complexes in targeting of receptors for down-regulation also in mammalian cells.

The endosomal intermediates between sorting and late endosomes, in which small vesicles are enclosed within an endosomal membrane, are called multivesicular bodies (MVBs)[49,83]. MVBs are transported along microtubules towards late endosomes, with which they eventually fuse[4]. In contrast to MVBs that are typically spherical, late endosomes are highly pleiomorphic with cisternal, tubular and multivesicular regions. Also their protein/lipid composition is distinct from that of MVBs. Late endosomes also function as an important sorting station in the endocytic pathway. The mannose-6-phosphate receptor cycles from late endosomes back to the Golgi network[39] whereas molecules of class II major histocompatibility complex (MHC) are transported from late endosomes to the plasma membrane in maturing dendritic cells[25]. Receptors in the degradative endosomal pathway are sorted from late endosomes to lysosomes. Along the endocytic pathway, the intravesicular pH drops from pH 6.0-6.5 in sorting endosomes to pH 4.5-5.5 in late endosomes and the pH reaches 4-5 in lysosomes. Both the low internal pH and the degradative enzymes within the membrane-bounded organelles make the lysosomes the site of degradation of proteins in the endocytic pathway.

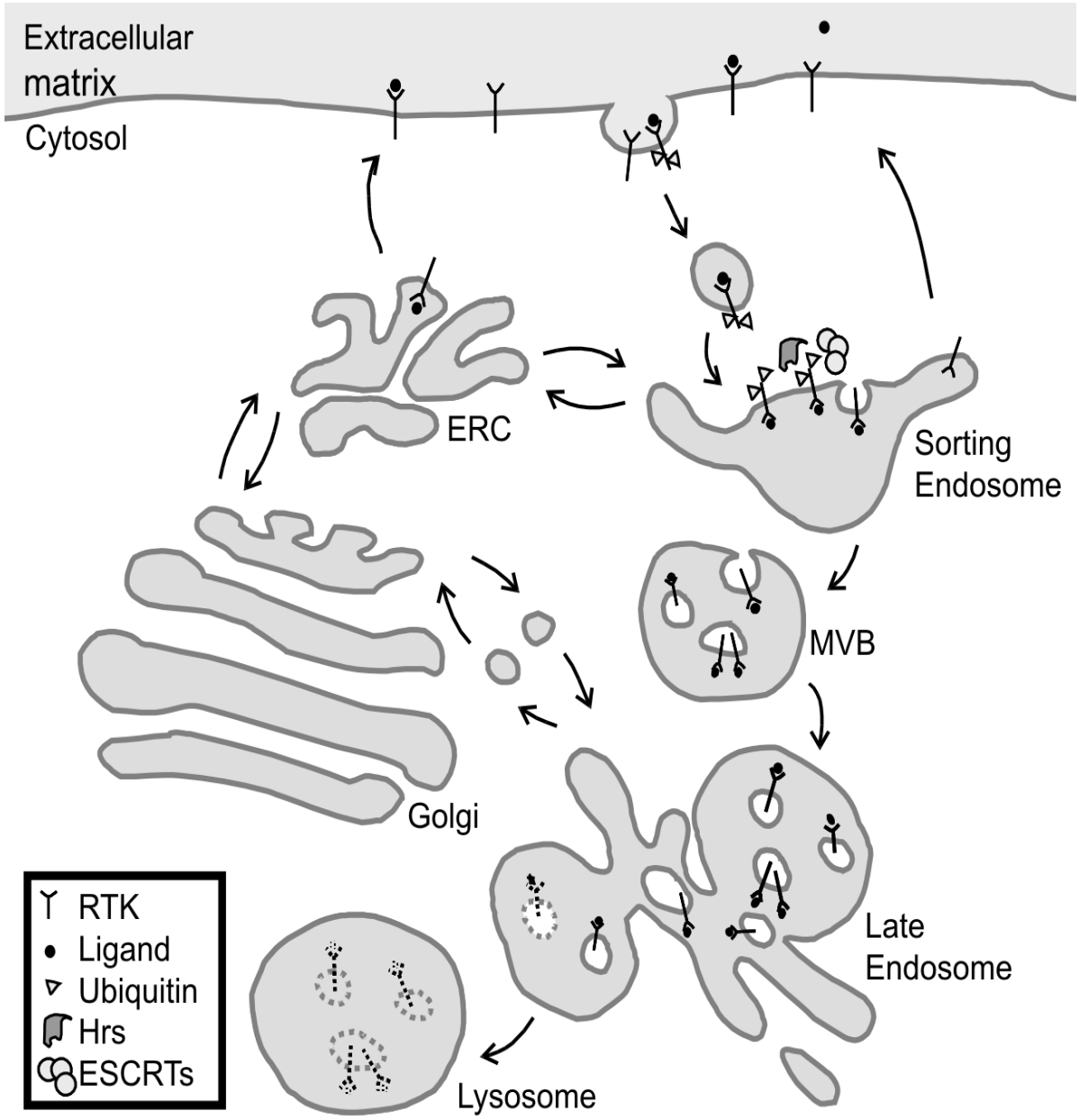


*Figure 1.* ***Receptor mediated endocytosis.*** *Upon internalization the receptors appear in sorting endosomes. At the sorting endosomes, receptors that are destined to be degraded in the lysosomes become ubiquitinated, recognized by Hrs and the ESCRT complexes and invaginated into the endosomes. Receptors that are not retained in the sorting endosomes recycles either directly or via the endocytic recycling compartment, ERC, back to the cell surface. Endosomes containing proteins in internal vesicles are referred to as multivesicular bodies, MVBs. MVBs fuse with late endosomes and the endocytosed material is then sorted from the late endosomes to the lysosomes. Materials are also transported between the Golgi apparatus and the late endosomes and between the Golgi apparatus and the ERC.*

The role of RTK endocytosis might extend beyond controlling signalling activity at the cell surface. It has become clear in recent years that many activated tyrosine kinase-coupled transmembrane receptors continue to propagate signals after internalization, and that lysosomal degradation may be required to terminate signalling[131]. Classical studies of endocytosis of low-density lipoprotein (LDL) implied that the ligand dissociates from the receptor in the acidic environment of endosomes so that the ligand and the receptor are sorted differently[42]. However, it was found that receptor-ligand complexes of RTKs, such as EGF, platelet-derived growth factor (PDGF) and nerve growth factor (NGF), do not significantly dissociate at endosomal pH[129,132,146]. Consequently, a great amount of these receptors remain ligand-bound in endosomes. The preservation of ligand-receptor complexes results in the existence of a pool of receptors that remains dimerized and thereby potentially active. Tyrosine phosphorylation and kinase activity of internalized receptors was first shown for EGF and insulin receptors and later reported for other RTKs[6,129]. RTK mediated signal transduction is accomplished by cascades of protein-protein interactions. Consistent with the presence of phosphorylated internalized receptors, several receptor-interacting proteins are found in endosomes[131].

It has also been reported that the intracellular part of EGF receptors can be proteolytically cleaved under binding of ligand and that fragments of the receptors can then be transported directly to the nucleus[95]. The more controversial observation that full length tyrosine kinase receptors can travel from the plasma membrane to the nucleus and possibly signal inside the nucleus has been reported from several groups[78,141]. In addition, exogenous growth factors have also been found to accumulate in the nucleus or in the cytosol[3,79,97,115,142]. However, the process of translocation and the possible function of growth factors, receptors and fragments of the receptors in the nucleus are still poorly understood.

## 4.3 Fibroblast growth factors

Genes, transcripts and proteins of the fibroblast growth factor superfamily have been identified in invertebrates as well as in vertebrates[24,94]. Defining features of the family are a strong affinity for heparin and heparan sulfate[15], and a highly homologous central core of 120 amino acids[148]. The family comprises 22 structurally related polypeptides in humans[100,108] encoded by distinct but evolutionary related genes[34,99], ranging in molecular mass from 14 to 34 kDa and sharing 13-71% amino acid identity[100]. Further diversity in the protein family is generated through the use of alternative translation initiation sites within the messenger RNA[31] as well as alternative splicing[103,139,150]. The crystal structure of the prototypic FGF family members, FGF1 and FGF2 has been shown to consist of twelve antiparallel β strands arranged to form a cylindrical β-barrel closed by the more variable amino- and carboxy-terminal streches[148].

FGF1, FGF2, FGF9 and FGF11-14[1,61,84,127], as opposed to other FGFs, lack a signal sequence required for secretion through the classical endoplasmic reticulum-Golgi apparatus pathway. FGF11-14 are believed to remain intracellular[127], while FGF1, FGF2 and FGF9 are released from the cells by a mechanism different from the classical ER-Golgi secretory pathway. Secretion of FGF1 is elevated under several stress conditions, such as hypoxia, serum starvation or heat[60,91,124]. Released FGF1 is unable to bind heparin and exist as an inactive homodimer in complex with the calcium binding protein, S100A13 and the extravesicular domain of synaptotagmin, a transmembrane component of synaptic vesicles[75,136]. Despite the structural and functional similarity between FGF1 and FGF2, the peptides may utilize distinct ER-Golgi independent secretory pathways. FGF2 is not secreted in response to heat shock and vesicle shedding has been proposed as a possible secretion mechanism[82,137]. Recently, there was reported that secretion of FGF2 did not require protein unfolding[7].

Accumulating evidence indicates that FGF1, FGF2 and FGF3 can act intracellularly as well as extracellularly[12,58,66]. Exogenous FGF1 and FGF2 are able to translocate to

the cytosol and the nucleus. Evidence for membrane translocation of exogenous FGF1 and FGF2 have been obtained by farnesylation studies of a growth factor mutant that contains a C-terminal farnesylation signal, a CAAX-box. Since the farnesyl transferase is located only in the cytosol and the nucleus[22], farnesylation of an externally added CAAX containing protein indicates its translocation across the cellular membrane. This was demonstrated for both FGF1[142] and FGF2[79]. In another approach, phosphorylation of exogenous FGF1 by protein kinase C, an enzyme exclusively present in the cytosol and the nucleus, demonstrated membrane translocation of the growth factor[67]. Localization to the nucleus appears to depend on the presence of a nuclear localization sequence (NLS) within the growth factors[58,66,110]. It has been suggested that nuclear FGF1 is involved in a mechanism of regulation of DNA-synthesis[67]. However, the best described signalling mechanism induced by external FGFs is the signalling mediated through binding to high-affinity cell-surface receptors (FGFRs) that possess tyrosine kinase activity[121].

## 4.4 Fibroblast growth factor receptors

### 4.4.1 High-affinity FGF receptors (FGFRs)

The fibroblast growth factor receptor family comprises a variety of polypeptides encoded by five closely related genes. FGFR1-4 have a conserved overall structure, sharing up to 72% identity. FGFR1 and FGFR2 are most similar, while FGFR1 and FGFR4 are least similar[62]. The fifth member of the gene family does not contain a tyrosine kinase domain but still share 32% identity within the extracellular part with the other FGF receptors[126]. Additional diversity among the receptors is generated by alternative splicing of receptor 1-3, resulting in a variety of receptor isoforms[62].

The prototypical FGF receptor consists of an extracellular domain, a single transmembrane domain and an intracellular domain[62] (Figure 2). The extracellular domain contains two or three immunoglobulin like domains (D1-D3), dependent on alternative splicing. Between D1 and D2 is a unique acidic region referred to as the

acidic box. D2 contains a heparin binding domain[64]. In addition, a signal peptide at the amino-terminus is cleaved off after translocation of the newly synthesized receptor into the endoplasmic reticulum. The intracellular part of the receptor contains a juxtamembrane stretch, a split tyrosine kinase domain and a C-terminal tail.

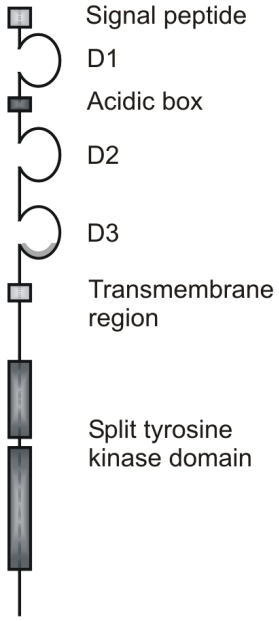


*Figure 2.* ***The prototypical FGFR.*** *The membrane proximal half of D3 is indicated in grey*

Different exon usage results in receptors which may be truncated, lack immunoglobulin like domains, or utilize different coding regions for the same Ig-like domains. One of the most important mechanisms by which FGFRs determine specificity for different FGFs is by alternate exon usage of the membrane proximal half of the D3. The exons encoding the membrane proximal half of D3 are designated IIIa, IIIb and IIIc. Such alternative splicing events are regulated in a tissue-specific manner. Usually, the expression of version IIIb is restricted to epithelial cells and IIIc to mesenchymal cells [8,101].

### 4.4.2 Low affinity FGF receptors

A common feature of FGFs and several other growth factors is a remarkable affinity for heparin and cell-surface heparan sulfate proteoglycans (HSPGs). Heparin and heparan sulfate are common glycosaminoglycans in proteoglycans. Heparan sulfate contains low and highly sulfated sites while heparin is more uniformly highly sulfated[34]. Unlike cell-surface tyrosine kinase receptors, HSPGs are not able to transduce any signal, but they can function as modulators of growth factor activities[119].

Binding of FGFs to HSPGs presented at the cell surface and in the extracellular matrix protects them from inactivation by heat and acid[46]. Another effect of HSPG binding in the extracellular milieu is the protection of the growth factors from proteolytic degradation by circulating proteases such as trypsin and plasmin[120,128].

Binding of FGFs to HSPGs also creates a local reservoir of FGFs that can be released by extracellular enzymes capable of degrading the proteoglycans[59]. Furthermore, binding of FGFs (and FGFRs) to heparins/HSPGs plays an important role in the formation of stable FGF-FGFR complexes at the cell surface[57].

### 4.4.3 Cysteine-rich FGF receptor

Various FGFs bind with high affinity also to a cysteine rich FGF receptor (CFR)[16]. The CFR lacks tyrosine kinase activity and does not belong to the FGFR family. Although reports indicate that CFRs are involved in intracellular regulation of FGF secretion[70] their function are generally unknown. CFR binds FGF in a heparin independent manner, but exhibit high affinity for HSPGs[149]. The full-length form of CFR is primarily located in the Golgi apparatus, while a proteolytically cleaved derivative has also been found secreted and deposited in the extracellular matrix[43].

## 4.5 Signalling from high-affinity fibroblast growth factor receptor

FGF binding to and dimerization of the specific tyrosine kinase receptor is a more complex process than in the case of other growth factors. FGF binds the FGFR in the D2-D3 junction, and heparin is involved in bridging and stabilizing two FGF/FGFR complexes in a receptor dimer[106,107,123]. Although HSPGs generally are required for formation of stable FGF/FGFR signalling complexes, activation of FGFRs by FGFs in the absence of HSPGs has also been reported[29]. Two forms of the growth factor/receptor-signalling complex can then exist, a less stable FGF/FGFR (2:2) complex and a more stable FGF/FGFR/HSPG (2:2:2) complex with prolonged signalling activity[99]. The FGF/FGFR/HSPG complex has also been proposed to assemble around one central heparin molecule, linking two FGFs into a dimer that bridges between two receptor chains (2:2:1)[104]. Since FGFRs also contain a heparin-binding site in D2 involved in formation of the signalling complex[64,107], it is not excluded that heparans could induce FGFR dimerization and activation without the

growth factor[35]. It has been proposed that D1 and the acidic box between D1 and D2 act cooperatively to negatively regulate FGFR function by competing with FGF and heparin for FGFR binding[96,122]. In this way D1 and the acidic box might play an autoinhibitory role, regulating binding of heparans and FGF to FGFR.

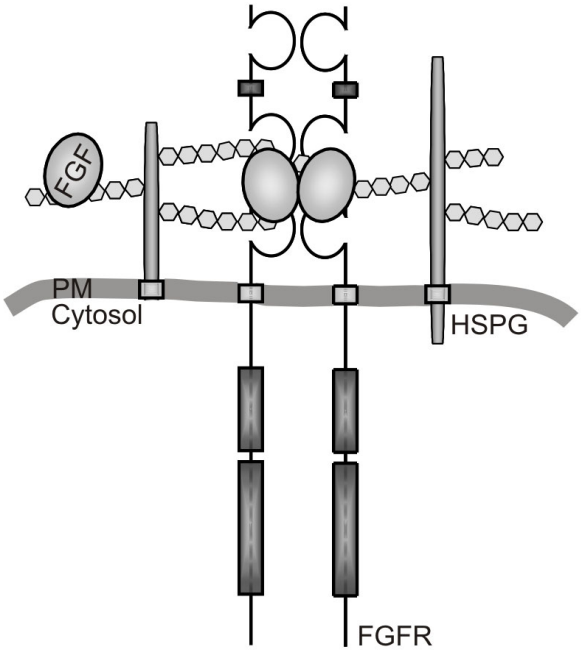


*Figure 3.* ***FGFR activation***

The dimerization of FGFRs by FGFs results in activation of the intrinsic tyrosine kinase and autophosphorylation of tyrosine residues in the intracellular part of the receptor. The phosphorylated tyrosine residues serve as binding sites for SH2 and PTB domain-containing signalling molecules. These molecules often possess an enzymatic activity (PLC-γ)[14,85] or are adaptor molecules (Shc, FRS2, Shb, Crk)[69] that associate with other signalling enzymes which in turn are either positive or negative regulators of FGF signalling[50,73,73,76,143] (Figure 4).

PLC- γ/PKC, PI 3-Kinase/Akt and Ras/MAPK are three major downstream signalling pathways activated by FGFs[11] (Figure 4). Grb2, a small adaptor protein, binds directly to FRS2 or Shc, both of which are phosphorylated on tyrosines by the activated FGFR, and recruits the nucleotide exchange factor, Sos to the plasma membrane. Sos catalyzes the exchange of GDP for GTP on Ras and thereby promotes the activation of Ras and the MAP kinases, Erk1 and Erk2 downstream of Ras[69]. PI 3-kinase seems to be directly bound to Gab1, which is also recruited by Grb2 to the FRS2/receptor complex[98]. Activated PI 3-kinase phosphorylates phosphatidylinositol 4,5 bisphosphate (PI(4,5)$P_2$) , generating phosphatidylinositol 3,4,5 trisphosphate

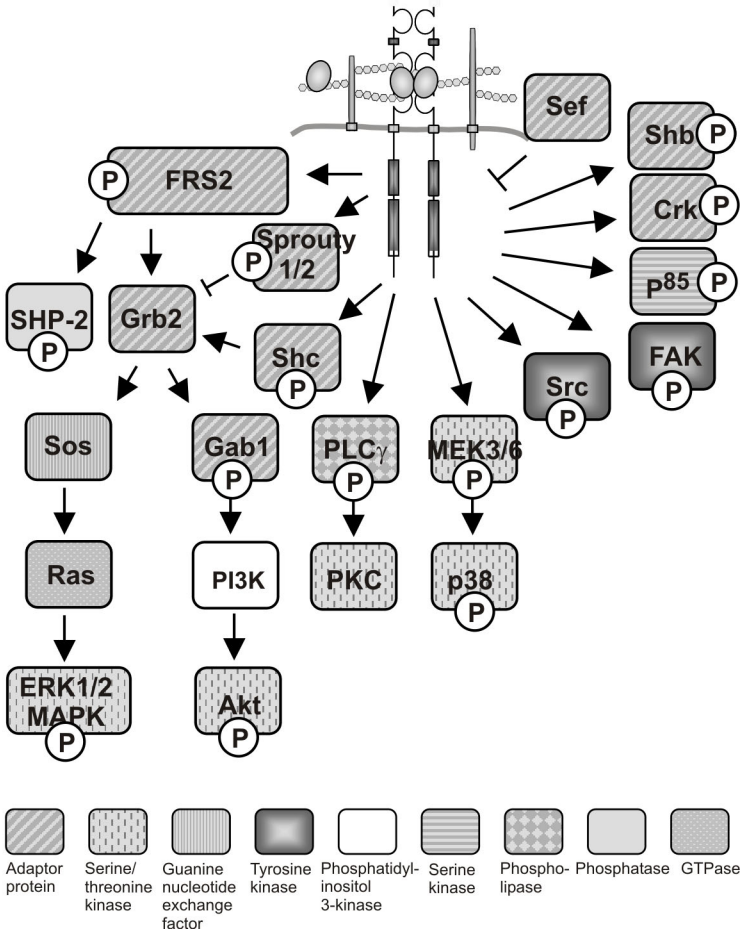


*Figure 4.* ***Signalling pathways activated by FGFRs***

($PI(3,4,5)P_3$). The $PI(3,4,5)P_3$ serves as a docking site for signalling proteins such as Akt[121]. It has also been reported that FRS2 could link FGFR activation to atypical PKC isoforms[77]. PLCγ on the other hand binds directly to the FGFR and becomes tyrosine phosphorylated and active upon binding[14,85], leading to hydrolysis of $PI(4,5)P_2$ to inositol 1,4,5 trisphosphate ($IP_3$) and diacylglycerol. $IP_3$ generation leads to release of $Ca^{2+}$ from internal stores, whereas diacylglycerol activates members of the protein kinase C family (PKC)[121].

When Grb2 is bound to Sprouty1 and Sprouty2, which are translocated to the plasma membrane and become phosphorylated upon FGF stimulation, the recruitment of Grb2-SOS to FRS2 is inhibited[53]. The transmembrane protein Sef was found to inhibit FGF induced proliferation by interaction with the FGFR[74]. The

phosphotyrosine phosphatase SHP-2, activated through binding to FRS2 is involved in regulation of signal transduction downstream of tyrosine kinases[69].

Several cytoplasmic kinases are also activated or inactivated by FGFR stimulation. A serine kinase, p85, has been shown to associate with activated FGFR4, implicating a role for serine phosphorylation in signal transmission by the receptor[138]. FGFR1 can stimulate or inhibit the Src kinase activity in a PKC-dependent manner[69] and the kinase is involved in a variety of signalling cascades in FGF stimulated cells[144]. FAK another cytoplasmic tyrosine kinase is also activated by FGF stimuli[105]. The adaptor proteins Crk and Shb are phosphorylated on tyrosines by the activated FGFR and provide additional docking sites for downstream signalling molecules[69].

The signalling pathways activated by FGFRs seem to include various signalling molecules. However, further investigations will be necessary to reveal the complete picture of FGFR signalling and the specificity of the induced signals.

## 4.6 Endocytosis and intracellular sorting of the FGF/FGFR complex

FGF is rapidly internalized after binding to its high-affinity receptor[29,33,81]. The endocytic process of FGFs/FGFRs has been described in a few studies and it appears that the different FGFRs and their isoforms may take different pathways and that this also may vary between different cell types.

FGF7 (KGF) bound to FGFR2 (IIIb) (KGFR) has been shown to be taken up by clathrin mediated endocytosis in NIH/3T3 cells stably transfected with KGFR, as well as in A253 carcinoma cells and in human cultured karatinocytes[81]. However FGF1/FGFR4 was reported to be endocytosed mainly by a mechanism different from the clathrin mediated pathway and caveolae in COS cells[21]. In HeLa cells FGF1/FGFR4 was reported to be endocytosed partly by a clathrin dependent pathway, partly by a non-clathrin/non-caveolae mechanism[20]. FGF2 has been reported to be endocytosed mainly through caveolae in BHK cells and ABAE cells

expressing endogenous FGF receptors[38]. The signals within the FGFR that mediate endocytosis are not well defined, but phosphorylation events induced by the tyrosine kinase appear to be important for efficient endocytosis[20,92,133].

Irrespective of their mechanism of internalization, after endocytosis, the FGF/FGFR complexes are shown to enter early endosomes/sorting endosomes[21,38]. Subsequent to their presence in sorting endosomes, FGF7/FGFR2 (IIIb) was found to be sorted to late endosomes in HeLa cells[9] and FGF2 has also been shown to be sorted to late endosomes/lysosomes in BHK cells[38]. On the other hand, FGF1/FGFR4 was sorted mainly to the recycling compartment in COS cells and in HeLa cells[21]. This transport was apparently regulated by the receptor kinase, as a kinase dead mutant of FGFR4 showed increased transport to lysosomes[20].

Degradation of internalized FGF receptors has been observed after a few hours[9,90,133]. It has been shown that binding of FGF to FGFR1 and FGFR3 induces ubiquitination of the receptors and that this contributes to down-regulation of the receptor[86,87,143]. The FGFR was found to recruit the ubiquitin ligase Cbl by an indirect mechanism involving the docking protein FRS2 and Grb2[143]. Activated FGFR3 has recently been reported to be targeted for lysosomal degradation through c-Cbl-mediated ubiquitination while FGFR3 harbouring mutations associated with *achondroplasia* and *thanatophoric dysplasia types II* (TDII) has been reported to escape lysosomal targeting[19].

RTK signalling does not only occur at the plasma membrane, but also from internalized ligand/receptor complexes. FGF7/FGFR2 were found to remain associated in active complexes through the endocytic pathway[9,81], and activated FGFR4 was found in the recycling endosomal compartment[21].

Internalized FGF1 is unusually long lived[10,89]. In various cell types only 10-30% of the internalized growth factor was found to be degraded after 6-8 hours[20,92] and FGF1 can still be detected 24 hours after internalization[32,48].

Accumulating evidence for cell-entry of exogenous FGF1 and FGF2 indicates that they can act intracellularly as well as extracellularly[97]. FGF1 and FGF2 activate not only the cell-surface receptors, but in addition, the receptor-bound growth factor is endocytosed and translocated across the membrane to reach the cytosol and the nucleus[67,79,142]. Recently obtained data have shown that translocation of the growth factor to the cytosol occurs from the lumen of intracellular vesicles and that the translocation process of internalized FGF1 requires electric potential across the vesicular membrane, generated by vacuolar proton pumps[80]. Translocation of FGFs has been reported in NIH/3T3 cells, HUVE cells, CPAE cells expressing endogenous FGFRs[80], and in COS cells transiently transfected with FGFR4[68].

It has also been reported that FGF2 stimulation induces nuclear translocation of FGFR1[78]. The nuclear import of FGFR1 is mediated by importin β, and was found to play a role in the regulation of the cell cycle[41,117]. It has been suggested that the association of the FGFR1 transmembrane region with the ER membrane could be relatively unstable and that the nucleus-destined receptor could be released from the ER/Golgi membranes into the cytosol before delivery to the plasma membrane[93]. However, receptors that have been present at the cell surface have also been reported to be transported into the nucleus.

Binding of FGFs to surface HSPGs can also leads to FGF internalization[118]. FGF1 and FGF2 internalized by binding to HSPGs was shown to be sorted to lysosomes[20,37].

## 4.7 Biological function of fibroblast growth factors

The first members of the FGF family were discovered in brain and pituitary extracts due to their growth-promoting activity on fibroblasts[5,45]. This activity turned out not to be specific for cultured fibroblasts, but could be observed on a variety of cell lines[47]. The list of biological activities attributed to FGFs has also been considerably extended and their ability to mediate a wide variety of biological responses is

probably the most remarkable feature of FGFs[135]. Biological responses to FGF signalling are depending on the target cell type, tissue context and the environment of the site where the signalling occurs.

FGF receptors can stimulate or inhibit cell proliferation depending on cell type. In mesoderm and ectoderm derived cells FGF signalling stimulate cell proliferation[47], whereas proliferation is inhibited by FGF signalling in chondrocytes[116]. Nuclear localization of FGF3 showed inhibitory effects on cell growth in mammary epithelial cells[66]. FGF stimulation can also induce apoptosis in certain cases. The induction of cell death by exposure to FGF2 is associated with a $G_1$ cell cycle arrest and activation of initiator and effector caspases[13]. On the other hand FGFs have also been shown to delay apoptosis in various cell lines by upregulated expression of the antiapoptotic protein, bcl-2[71]. FGFR signalling is found to be critical for cell migration as cells introduced to a dominant negative mutant of FGFR1 were unable to migrate[102]. FGF signalling is also implicated in cell differentiation[26,47,50].

FGFs play important roles in development[40]. They are well known inducers of mesoderm[62] and they have also been shown to be relevant in organogenesis, particularly in that of the nervous system, the lungs and the limbs[108]. FGFs are also believed to be important in wound healing, in which formation of new blood vessels is a significant part of the process[15,33].

## 4.8 FGF implicated in human disorders

Up-regulation of FGFs/FGFRs and structural alterations in genes encoding FGFRs are found in a number of human cancers, and FGFs and the FGF signalling pathways appear to play important roles in tumour development and progression. The growth factor can directly promote tumour cell growth due to their mitogenic, antiapoptotic and angiogenetic activity[33,108]. Angiogenesis, the formation of new blood vessels from existing ones, is required for a tumour to grow beyond the size where diffusion of nutrients and waste products can keep the tumour cells alive. Angiogenesis is also

crucial for metastatic progression. FGF1 and FGF2, together with vascular endothelial growth factors, are considered as the most common tumour angiogenic factors[147].

Dysregulation of FGF signalling in cancer arising from an increased availability of FGFs is a result of overexpression of different FGFs or uncontrolled release of FGFs sequestered in the extracellular matrix[108]. FGFs have been shown to be up-regulated in various forms of cancer such as human pancreatic cancer, breast cancer, renal cancer and some prostate cancers[52,72]

Dysregulation of FGF signalling as a result of alterations at the level of the receptor has been shown to occur through inappropriate expression, point mutations, splice variations and genomic alterations[108]. FGFRs have been found overexpressed in several human tumour samples such as brain tumours, breast tumours, pancreatic cancer and prostate cancer[36,72,88,145]. The tumour promoting effect of FGFR overexpression seems to depend on the target cell type and tissue context. In prostate cancer, overexpression of FGFR1 accelerates tumorgenesis whereas FGFR2 in these tumours inhibit malignant progression[30]. Mutations in FGFRs resulting in constitutively active forms of the receptors have also been mapped in several human cancers. Activating mutations in FGFR3 have been implicated in human multiple myeloma and bladder cancer[18,125]. Alternative spliced forms of the receptors may also play significant roles in human cancers. A soluble, spliced variant of FGFR3 is found frequently expressed in tumour cells and tissues such as neuroblastoma, bladder carcinoma, breast carcinoma, colorectal carcinoma and tumours of the Ewing's sarcoma family and appears to contribute to malignant transformations[134] and a N-terminally truncated isoform of FGFR4 have been implicated in pituitary tumorgenesis in a majority of human pituitary adenomas[109].

A large number of human skeletal disorders such as various forms of dwarfism have been mapped to mutations in genes encoding FGFR1-3[28]. These are syndromes where either growth of the long bones is affected (*chondrodysplasia*) or fusion of the cranial structures is premature (*craniosynostosis*). Common for most of the FGFR mutations

found in these disorders are that they cause ligand-independent activation of the receptor kinase. In several cases the mutation creates a free cysteine-residue in the extracellular part of the receptor, believed to form intermolecular disulfide bonds and promote dimerizing and thereby ligand-independent activation of the receptor tyrosine kinase[140]. Mutations in the transmembrane domain are thought to facilitate intermolecular hydrogen bonds, also leading to ligand independent receptor dimerization[140]. Directly activation of the receptors through mutations in the kinase domain is also found in several forms of skeletal disorders[140]. Enhancement of FGF binding affinity[56] and escaping lysosomal targeting[19] are also observed properties of FGFRs harbouring skeletal disorder mutations.

## 4.9 Perspectives

The FGFs and their high-affinity receptors clearly play important roles in the development and growth of the organism. Since the signalling through FGFs appears to be fundamental in so many different processes and irregularities in FGF-mediated signalling are implicated in several serious disorders, the basic biology of FGFs, FGFRs and their signalling is of great interest. Much effort has concerned the elucidation of the biological responses to FGF signalling, whereas the cell biology of FGFs and their receptors is less studied. Knowledge about how growth factors and corresponding receptors function is crucial in order to try to stimulate or inhibit their effects for therapeutical purposes. Therefore, studies on the basic biology of FGFs, FGFRs and their signalling might lay the basis for future clinical applications.

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# 6. Manuscript

# Different intracellular trafficking of FGF1 endocytosed by the four specific FGF receptors

Ellen Margrethe Haugsten, Vigdis Sørensen*, Sjur Olsnes and Jørgen Wesche

The Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

The sorting of internalized fibroblast growth factor 1 (FGF1) was studied in HeLa cells transfected with either of the four spesific FGF receptors, (FGFR1-4). Fifteen min after endocytosis, externally added FGF1 bound to either receptor was localized in sorting/early endosomes. Subsequently, FGF1 internalized via FGFR1-3 localized mainly to late endosomes/lysosomes, in a similar way as endocytosed EGF. On the other hand, FGF1 internalized via FGFR4 was found to mainly follow the same intracellular pathway as the recycling ligand transferrin. Furthermore, FGF1 endocytosed by FGFR4 was more slowly degraded than FGF1 endocytosed by FGFR1-3. In addition, internalized FGFR4 as such was more slowly degraded than the other receptors. The data indicate that after endocytosis FGFR4 is sorted mainly to the recycling compartment while FGFR1-3 are sorted to degradation in the lysosomes. By aligning the amino acid sequence of the intracellular part of the four FGF receptors, several lysines that are conserved in FGFR1-3 but not in FGFR4 were revealed. Lysines are potential ubiquitination-sites and could thus target a receptor for sorting to lysosomes. Indeed, we found that FGFR4 is less ubiquitinated than FGFR1 after internalization, which is possibly the reason for the different sorting of the receptors.

* The experiment presented in Figure 6 was performed by Vigdis Sørensen.

## *Introduction*

Many growth factors and cytokines can bind to more than one receptor, but in many cases the different roles of the separate receptors in signal transduction are unclear. Intracellular sorting of ligand-receptor complexes may determine their signalling and we have here studied the cellular trafficking of ligand bound to receptors for fibroblast growth factors (FGFs).

The large family of FGFs comprises 22 structurally related heparin binding polypeptides which are involved in the regulation of various cellular responses in developmental and physiological processes[30]. The FGFs mediate their biological effects through binding to high-affinity cell-surface receptors, FGFRs. The FGFR family constitutes a variety of polypeptides encoded by four closely related genes[18]. The receptors share common structural features and consist of an extracellular ligand binding domain, a transmembrane domain and a cytosplasmic region. The extracellular domain contains a unique acidic region and two or three immunoglobulin like domains (D1-D3), dependent on alternative splicing. The cytoplasmic region contains a split tyrosine kinase domain[18].

Binding of FGFs to FGFRs is stabilized by heparan sulfate proteoglycans (HSPGs) and results in a dimer receptor-ligand complex that activates the intracellular tyrosine kinase domain by autophosphorylation. The autophosphorylation triggers the transient assembly of a large intracellular complex, which activates downstream signalling pathways such as PLC- γ/PKC, PI 3-Kinase/Akt and Ras/MAPK[2]. Depending on the target cell type, FGF signalling can induce cell proliferation, cell growth arrest, cell differentiation, apoptosis and cell migration[2,3,13].

Signalling from activated transmembrane receptors is attenuated by degradation in lysosomes. Lysosomal targeting of tyrosine kinase receptors is best illustrated for the epidermal growth factor receptor (EGFR) and involves the attachment of ubiquitin to lysine residues in the cytoplasmic tail of the activated receptor[33]. Upon internalization, the receptors appear in early/sorting endosomes where the receptors that are destined to be degraded in the lysosomes become ubiquitinated, recognized by Hrs and the

ESCRT complexes and internalized into the endosomes by membrane invagination[32]. Endosomes containing internal vesicles are referred to as multivesicular bodies, MVBs. MVBs fuse with late endosomes and the endocytosed material is then sorted from late endosomes to lysosomes where it is degraded.

Receptors that are not retained in the sorting endosomes recycle either directly or via the endocytic recycling compartment, ERC, back to the cell surface. Most receptors known to recycle possess no signalling activity and are often associated with uptake of nutrients. The transferrin receptor, TfR, is known to recycle via the ERC and is often used as a marker for the recycling endocytic pathway[42]. The transferrin receptor binds its ligand, diferric transferrin and is rapidly internalized. In the acidic environment of sorting endosomes the two iron ions are released from the ligand and transported into the cytoplasm, whereas the ligand/receptor recycles via the ERC to the cell surface. At the neutral extracellular pH, iron-free transferrin is released from the receptor[7]. The importance of ubiquitin as a signal for lysosomal sorting was illustrated when transferrin receptors fused to ubiquitin was found to be sorted into the degradative pathway[31].

From what is known about the endocytosis of FGFRs, it appears that they may utilize different mechanisms for internalization and that this also may vary between different cell types[5,6,11,22]. However, irrespective of the mechanism of endocytosis, FGF/FGFR complexes have been observed in early endosomes/sorting endosomes approximately 10 min after internalization[1,6,11]. Subsequent to their presence in sorting endosomes, KGF/KGFR (FGF7 and a splicing variant of FGFR2), was found to be sorted to late endosomes in HeLa cells[1]. FGF2 has also been observed in late endosomes and lysosomes in BHK cells[11]. On the other hand, FGF1/FGFR4 in COS cells has been found to accumulate in a juxtanuclear region, identified as the recycling compartment[6]. It was found that binding of FGF to FGFR1 and FGFR3 induces ubiquitination of the receptors and that this contributes to their downregulation[4,23,24,40]. Activated FGFR3 has recently been reported to be targeted for lysosomal degradation through c-Cbl-mediated ubiquitination while FGFR3 harbouring mutations associated with skeletal disorders were found to be less ubiquitinated and escape lysosomal targeting[4].

In order to compare the intracellular fate of the four related FGFRs upon internalization, HeLa cells transfected with either of the four FGFRs, were chosen as a model system, and FGF1, which binds equally well to the four FGFRs[29] was used as a ligand. The present work demonstrates that the four receptors are indeed sorted differently and that different levels of ubiquitination appear to be the molecular mechanism responsible for the different sorting.

## *Materials and methods*

### Materials

*Antibodies:* Rabbit anti-FGFR1, anti-FGFR2, anti-FGFR3 and anti-FGFR4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against tyrosine 653/654 phosphorylated FGFRs were from Cell Signalling (Beverly, MA). Mouse anti-EEA1 antibodies were obtained from Transduction laboratories (Lexington, KY) and mouse anti-LAMP-1 antibodies were from Developmental Studies Hybridoma Bank, (Iowa City, IA). Mouse anti-myc antibodies were from 9E10 hybridoma (Evan, Lewis, Ramsay 1985). Mouse anti-TfR antibodies were from Boehringer Mannheim (Mannheim, Germany). The secondary antibodies Cy2-conjugated anti-mouse IgG, Cy2-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were from Jackson Immuno-Research Laboratories (West Grove, PA).

*Chemicals:* Cy3-maleimide, Cy5-monofunctional reactive dye, heparin-Sepharose, streptavidin-Sepharose, ECL plus Western blotting system were from Amersham Biosciences (Buckinghamshire, UK). APS, TEMED, 40% Acrylamide/Bis-acrylamide and Restore Western Blot Stripping Buffer were from BIO-RAD (Hercules, CA). Fugene 6 was from Boehringer Mannheim (Indianapolis, IN). DMEM, streptomycin and penicillin were from GIBCO, Invitrogen (Carlsbad, CA). Alexa 488 EGF was from Molecular Probes, Invitrogen. Restriction enzymes were from New England Biolabs, (Beverly, MA). Mowiol was from Novabiochem Corporation (La Jolla, CA). Fetal calf serum was from PAA Laboratories GmbH (Linz, Austria). Easytag Methionine L-[$^{35}$S] was from Perkin Elmer (Boston, MA). Leupeptin to use on live

cells was from Peptide Institute Inc (Osaka, Japan). Ez-link sulfo-NHS-LC-Biotin was from Pierce (Rockford, IL). Rabbit Reticulocyte Lysate System was obtained from Promega Corporation (Madison, WI). Complete EDTA free Protease Inhibitor cocktail tablets were from Roche Diagnostics (Penzberg, Germany). T7 RNA polymerase was from Stratagene (La Jolla, CA). Other chemicals were from Sigma-Aldrich (St. Louis, MO).

*Equipments:* Cell culture plates were from BD Biosciences (San Jose, CA) and Nalge Nunc International (Rochester, NY). Immobilon-P PVDF membrane was from Millipore Corporation (Bedford, MA). QIAshredder columns were from QIAGEN (Hilden, Germany). Zeiss LSM 510 META confocal microscope and Zeiss LSM Image Browser (Version 3) were from Zeiss (Jena, Germany). Adobe Photoshop 7.0 was from Adobe (San Jose, CA). STORM gel and blot imaging system and Image Quant, Version 5.0 were from Molecular Dynamics, Amersham Biosciences (Buckinghamshire, UK). Chemi Genius Image Acquisition System was from Syngene (Camebridge, UK). Recombinant FGF1 was a generous gift from Dr. Antoni Wiedlocha, Institute for Cancer Research, The Norwegian Radium Hospital.

## Plasmids

pcDNA3-hFGFR1: cDNA encoding hFGFR1 IIIc was cut out from pSV7d[39] with *Eco*RI and *Xba*I in two fragments, and ligated into pcDNA3 (Invitrogen, Carlsbad, CA) cut with the same enzymes. pcDNA3-hFGFR2: cDNA encoding hFGFR2 IIIc lacking D1 was cut out from pBluescript (RZPD, Berlin, Germany, Clone ID: IMAGp998N0911701Q3) with *Not*I and *Spe*I, and ligated into pcDNA3 cut with *Not*I and *Xba*I. The pcDNA3-hFGFR3 construct was a generous gift from Dr. Avner Yayon, ProChon Biotech Ltd., Israel. The pcDNA3-hFGFR4 construct has been described earlier[19]. The pcDNA3-myc-tagged-ubiquitin was a generous gift from Dr. Harald Stenmark, Institute for Cancer Research, The Norwegian Radium Hospital. The pTriEX-2-FGF1 construct was a generous gift from Camilla Skiple Skjerpen, Institute for Cancer Research, The Norwegian Radium Hospital.

## Cells

HeLa cells were propagated in Dulbecco's Modified Eagle Medium, DMEM, supplemented with 10% (vol/vol) fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% $CO_2$ atmosphere at 37°C.

## Transfections

Transient expression of the different receptors was performed by transfecting HeLa cells with the plasmid DNA (pcDNA3 with appropriate inserts) by using Fugene 6 transfection reagent according to the manufacturer's protocol. Cells were seeded into plates the day preceding the transfection and experiments were performed 15-24 hours after transfection.

## Laser scanning confocal microscopy

Following the manufacturer's protocol, FGF1 was labelled with Cy3-maleimide and transferrin was labelled with Cy5-monofunctional reactive dye. Transiently transfected HeLa cells grown on coverslips at 37°C were incubated with Cy3-FGF1 for two hours at 4°C in the presence of 50 U/ml heparin in HEPES medium. The cells were then washed three times in PBS and incubated in DMEM with 0.3 mM leupeptin at 37°C for different periods of time. The cells were fixed in 3% paraformaldehyd in PBS for 15 min, washed three times in PBS and mounted in Mowiol. In some cases the cells were also incubated with Alexa 488 EGF, Cy3-FGF and Cy5-transferrin in the presence of 50 U/ml heparin and 0.3 mM leupeptin. When antibodies were used to visualize structures within the cell, the fixation was quenched with 50 mM $NH_4Cl$ in PBS for 15 min and the cells were permeabilized with 0.05% saponin in PBS for 5 min. The cells were then incubated with primary antibody in 0.05% saponin in PBS for 20 min, washed three times in 0.05% saponin in PBS and incubated with the secondary antibody coupled to a fluorophore. After washing once in 0.05% saponin and twice in PBS, the cells were mounted in Mowiol and examined with a Zeiss LSM 510 META confocal microscope. Images were prepared with Adobe Photoshop 7.0 and Zeiss LSM Image Browser.

## Quantification of colocalization

Images of transfected, randomly chosen cells were divided into squares, and every fifth square within the chosen cell was examined. Red structures indicating internalized Cy3-labelled FGF1 were compared with structures of the different markers and the proportion of red structures that colocalized with structures of the specific marker was calculated. The mean and standard deviation were calculated from 15 cells in each case.

## Degradation of internalized FGF1

[$^{35}$S]methionine-labelled 18 kDa, long form of FGF1 was produced by transcription from the pTriEX-2 plasmid using T7 RNA polymerase and translation in a rabbit reticulocyte lysate supplemented with Easytag Methionine L-[$^{35}$S] according to the manufacturer's protocol. HeLa cells, transiently transfected with the different FGFRs were incubated with [$^{35}$S]methionine-labelled 18 kDa form of FGF1 and 20 U/ml heparin at 37°C for 30 min to allow endocytosis via high-affinity receptors. Then the cells were washed twice with a high salt, low pH buffer (1 M NaCl, 20 mM NaAc, pH 4.0) and once with PBS on ice to remove excess and cell-surface bound FGF1. The cells were then either lysed immediately in lysis buffer (0.1 M NaCl, 10 mM $Na_2HPO_4$, 1% Triton X-100, 1 mM EDTA, supplemented with complete protease inhibitors, pH 7.4) or incubated further in growth medium with or without 100 μM chloroquine at 37°C for 3 or 6 h before lysis. [$^{35}$S]methionine-labelled FGF1 was extracted from the lysate by adsorption to heparin-Sepharose and analysed by 15% SDS-PAGE as described by Laemmli[20]. The proteins on the gels were fixed in fixative (25% methanol, 7.5% acetic acid) and then the gels were dried. Phosphorimager scanning and Image Quant, Version 5.0 software were used to estimate the relative amount of radioactive FGF.

## Degradation of internalized receptors

HeLa cells not transfected or transiently transfected with FGFR1, FGFR2, FGFR3 or FGFR4 were washed three times in PBS and cell-surface proteins were biotinylated with 0.5 mg/ml Ez-link sulfo-NHS-LC-Biotin in PBS for 15 min at 4°C. The

biotinylation reaction was quenched with 50 mM Tris-HCl pH 8.0. The cells were washed twice with PBS and then incubated for indicated periods of time in DMEM containing 100 ng/ml FGF1 and 20 U/ml Heparin. The cells were washed with PBS and lysed on ice in lysis buffer for 20 min. The lysate was centrifuged to remove nuclei and then biotinylated proteins were pulled down from the supernatant with streptavidin-Sepharose beads at 4°C over night. The beads were then washed three times in PBS containing 0.1% Tween 20 and finally resuspended in 15µl of reducing SDS-PAGE sample buffer. Proteins were separated by 7% SDS-PAGE and transferred to a PVDF membrane which was probed with anti-FGFR1, anti-FGFR2, anti-FGFR3 or anti-FGFR4 primary antibodies and HRP-conjugated secondary antibody. Immunoactivity was detected by using ECL plus Western blotting system and Chemi Genius Image Acquisition System. To compare the intensity of bands of interest on the membrane, ImageQuant software was used. Background correction was performed by subtracting values obtained by scanning adjacent areas of the membrane with the same size but containing no visible bands from those obtained with the bands of interest.

### Ubiquitination of internalized receptors

HeLa cells cotransfected with myc-ubiquitin and FGFR1, FGFR4 or empty vector were starved for 16 hours and then washed three times in PBS. Cell-surface proteins were biotinylated with 0.5 mg/ml Ez-link sulfo-NHS-LC-Biotin in PBS for 15 min at 4°C. The biotinylation reaction was quenched with 50 mM Tris-HCl pH 8.0 and the cells were washed twice with PBS. The cells were incubated for two hours in 200 ng/ml FGF1, 20 U/ml heparin and 0.3 mM leupeptin at 37°C in DMEM without serum. The cells were then washed once in DMEM without serum and lysed at 95°C for 5 min in 1% SDS in PBS. The lysate was decanted into QIAshredder columns and centrifuged two min at 4°C. Equal amounts of lysate and 2X pull down-buffer (2% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 40 mM NaF, 1% bovine serum albumine, 2 mM N-ethylmaleimide supplemented with protease and phosphatase inhibitors) were added to streptavidin-Sepharose beads to pull down biotinylated proteins. After tumbling one hour at 4°C the beads were washed twice in 1X pull-down buffer (0.5% SDS and 50% 2X pull-down buffer in PBS) once in 1:10

diluted PBS. The proteins that remained bound to the streptavidin-Sepharose beads were run on a 7% SDS-PAGE and then transferred to a PVDF membrane which was probed with anti-myc primary antibody and HRP-conjugated secondary antibody to detect the level of ubiquitination of internalized FGFRs. Immunoreactivity was detected using ECL plus Western blotting system and Chemi Genius Image Acquisition System. The membrane was stripped twice and reprobed with anti-phospho FGFR primary antibody and HRP-conjugated secondary antibody to detect the level of internalized receptors and anti-transferrin receptor primary antibody and HRP-conjugated secondary antibody to verify equal loading of the gel. To ensure equal expression of ubiquitin, the cells were analysed by immunofluorescence microscopy.

## *Results*

### Characterization of the endocytic pathway followed by FGF1/FGFR1-4

Upon ligand binding to the high-affinity FGF receptors, the ligand-receptor complexes are internalized[27,35] and transported to various intracellular compartments. Since FGF1 binds equally well to the four high-affinity FGF receptors[29], we have chosen this as a ligand and labelled it with the fluorescent dye Cy3. The fluorescent growth factor was used as a marker to explore the intracellular trafficking of the receptors. Cy3-labelled FGF1 has previously been shown to retain its binding capacity towards the FGFRs and HSPGs[6].

The distribution of fluorescent growth factor-receptor complexes was studied in HeLa cells transiently transfected with the different high-affinity receptors and incubated with Cy3-FGF1 for different periods of time. HeLa cells do not express detectable amounts of endogenous FGFRs. To avoid FGF1 binding to cell-surface heparan proteoglycans and to facilitate binding to the high-affinity FGFRs, heparin was added to the extracellular medium. The data in Figure 1 demonstrate that fluorescent FGF1 binds exclusively to the surface of transfected cells when treated with the growth factor at 4°C in the presence of heparin. When the cells were subsequently incubated

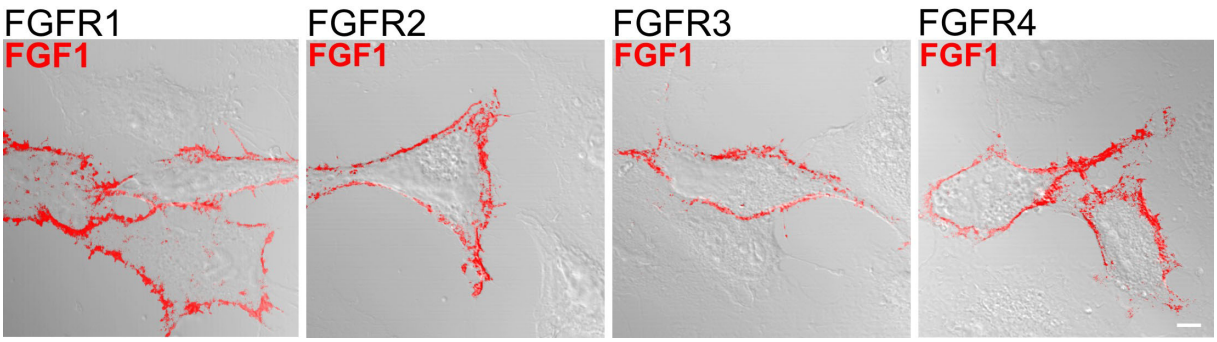



**Figure 1. Binding of FGF1 to cell-surface FGFRs.** HeLa cells were transiently transfected with FGFR1, 2, 3 or 4 and incubated with Cy3-labelled FGF1 and 20 U/ml heparin for two hours at 4°C. The cells were then fixed and examined by confocal microscopy. The red channel image was superimposed onto the corresponding interference contrast image. Scale bar, 5 µm.

at 37°C, the amount of growth factor at the surface was reduced and the fluorescent growth factor appeared as intracellular dots, indicating uptake into vesicles (Figure 2).

To determine whether FGF1 and the different FGFRs remain in the same compartments after internalization, we carried out double-labelling experiments where cells were allowed to take up Cy3-labelled growth factor for 2 h at 37°C in the presence of leupeptin (an inhibitor of lysosomal degradation) and then stained with antibodies against the different FGFRs. As shown in Figure 2, there was considerable overlap between internalized FGF1 and the specific fluorescent FGFR staining. This was demonstrated in the overlay experiments when spots labelled with both fluorophores appeared yellow. This finding indicates that the internalized growth factor remains bound to the receptor during the endocytic pathway as previously reported for FGF7/FGFR2[1,22] and FGF1/FGFR4[6].

To follow the endocytic pathway and to identify the intracellular structures where the different FGF1/FGFR complexes are localized upon internalization, the transiently transfected HeLa cells were allowed to bind Cy3-labelled FGF1 at 4°C and they were then incubated for different periods of time at 37°C. The cells were then fixed and stained with markers for different intracellular compartments. As shown in Figure 3, incubation for 15 min at 37°C resulted in good overlap of EEA1, a protein associated with early/sorting endosomes[26], and endocytosed Cy3-FGF1. Quantification of

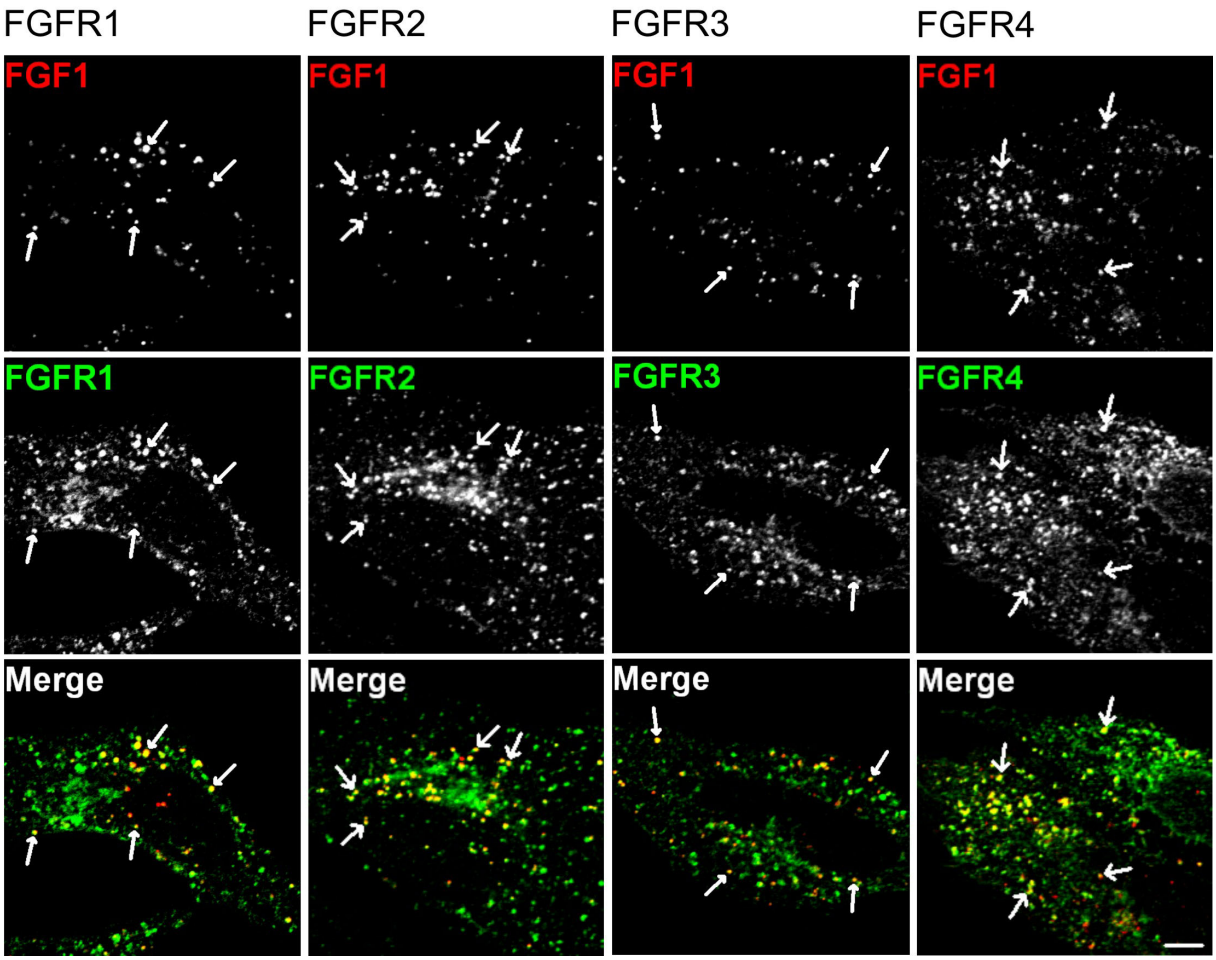



**Figure 2. Localization of FGFRs and endocytosed FGF1.** HeLa cells, transiently transfected with FGFR1, 2, 3 or 4 were incubated with Cy3-FGF1, 20 U/ml heparin and 0.3 mM leupeptin for two hours at 37°C. The cells were then fixed, permeabilized and treated with rabbit anti-FGFR1, anti-FGFR2, anti-FGFR3 or anti-FGFR4 primary antibodies. The cells were further treated with Cy2-conjugated anti-rabbit secondary antibodies and examined by confocal microscopy. Arrows point to colocalization. Scale bar, 5 µm.

colocalization revealed that the degree of overlap between the two fluorescent signals was similar for all the four high-affinity receptors, indicating that internalized FGF1/FGFR complexes reach the sorting endosomal compartment irrespective of receptor type (Figure 3B).

After a 2 h chase in the presence of leupeptin the major part of the internalized FGF1 in cells transfected with FGFR1-3 was found to colocalize with LAMP-1, a marker for late endosomes/lysosomes[10], while FGF1 in FGFR4 transfected cells was not (Figure 4A). About 90% of the FGF1 positive structures in FGFR1 transfected cells were LAMP-1 positive, whereas only around 45% of the FGF1 positive structures in cells

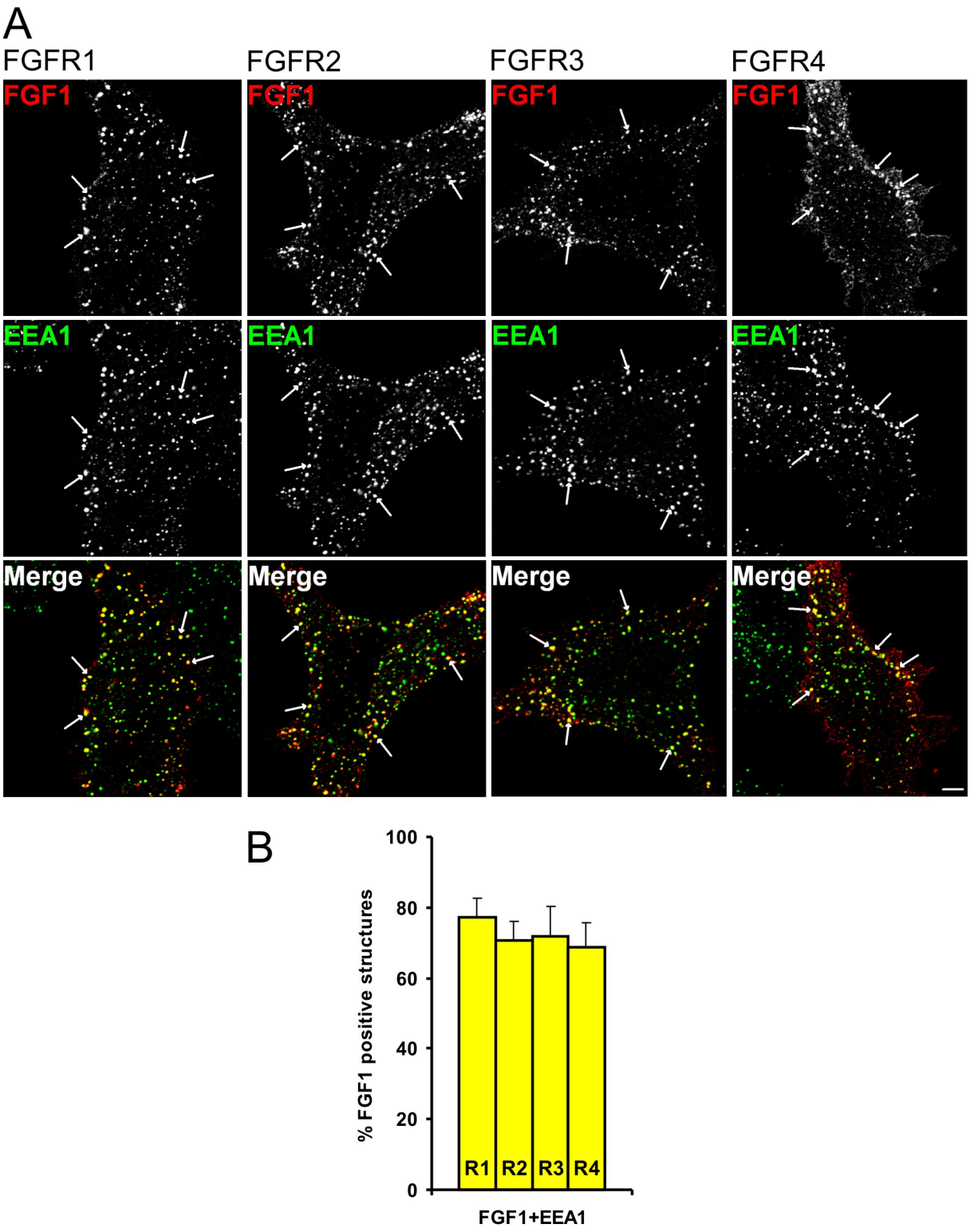



**Figure 3. Localization of EEA1 and endocytosed FGF1. (A)** HeLa cells were transiently transfected with FGFR1, 2, 3 or 4 and incubated with Cy3-FGF1 and 20 U/ml heparin for two hours at 4°C. The cells were washed and further incubated in the presence of 0.3 mM leupeptin for 15 min at 37°C. The cells were fixed immediately, permeabilized and treated with mouse anti-EEA1 primary antibody. The cells were further treated with Cy2-conjugated anti-mouse secondary antibody and examined by confocal microscopy. Arrows point to colocalization. Scale bar, 5 µm. **(B)** The percentage of FGF1 positive structures within cells transfected with FGFR1, 2, 3 or 4 that colocalizes with EEA1 was quantified as described in materials and methods. Error bars denote the standard deviation, n=15

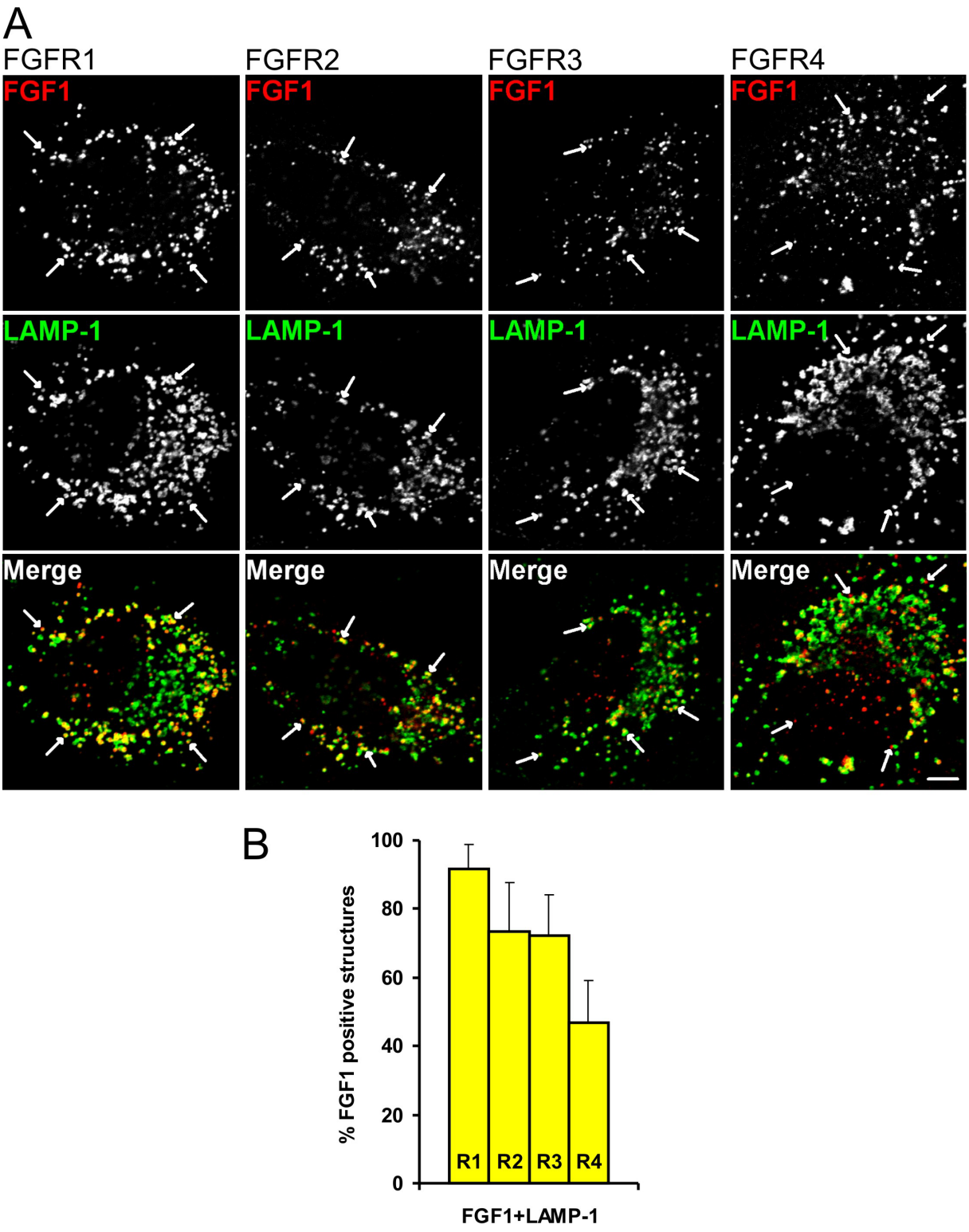


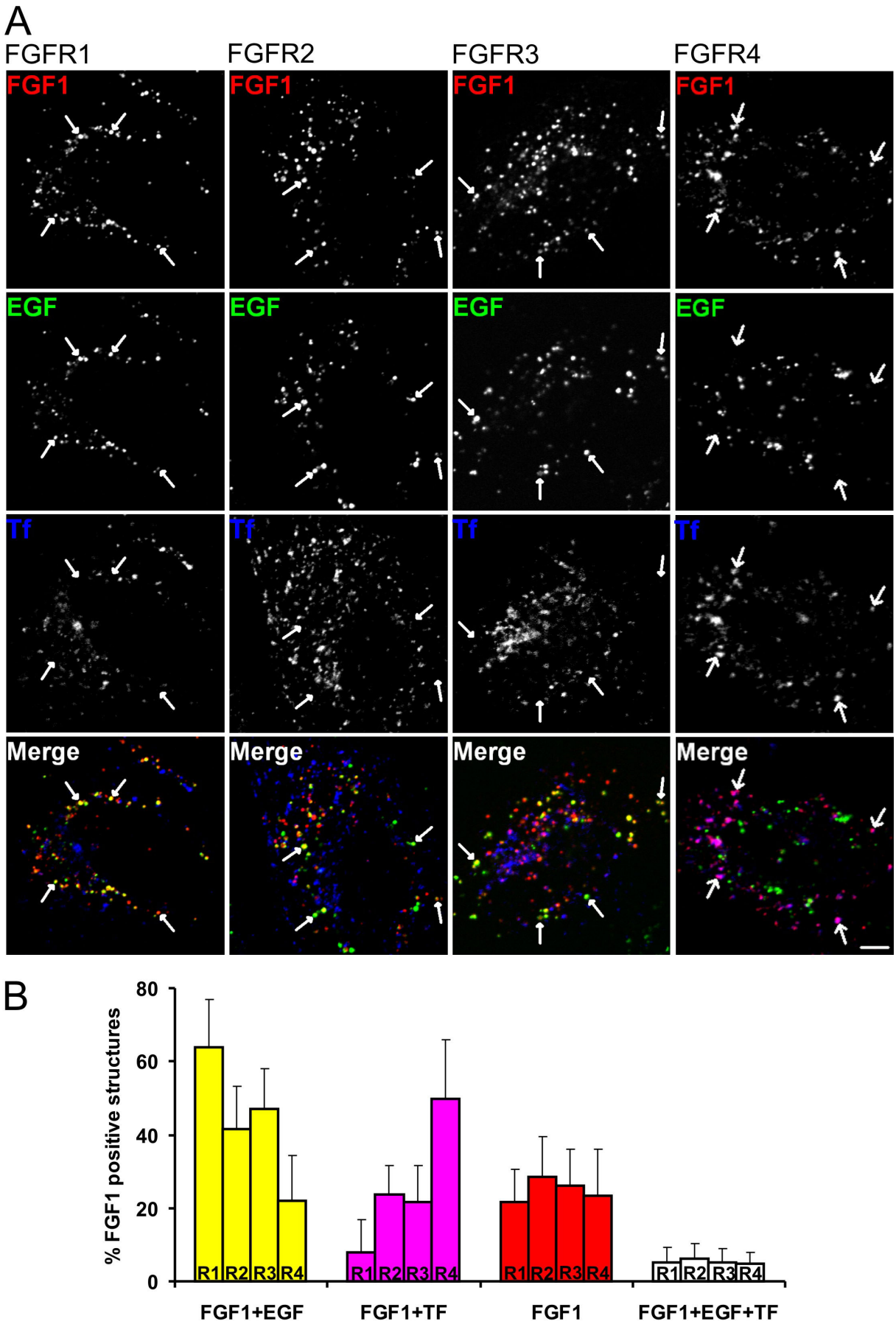

**Figure 4. Localization of LAMP-1 and endocytosed FGF1. (A)** HeLa cells were transiently transfected with FGFR1, 2, 3 or 4 and incubated with Cy3-FGF1 and 20 U/ml heparin for two hours at 4°C. The cells were washed and further incubated in the presence of 0.3 mM leupeptin for two hours at 37°C. The cells were fixed immediately, permeabilized and treated with mouse anti-LAMP-1 primary antibody. The cells were further treated with Cy2-labelled anti-mouse secondary antibody and examined by confocal microscopy. Arrows point to colocalization or lack of colocalization. Scale bar, 5 µm. **(B)** The percentage of FGF1 positive structures within cells transfected with FGFR1, 2, 3 or 4 that colocalizes with LAMP-1 was quantified as described in materials and methods. Error bars denote the standard deviation, n=15.

transfected with FGFR4 were LAMP-1 positive (Figure 4B). In the case of FGFR2 and 3 around 70% of the FGF1 positive structures were also positive for LAMP-1. These findings indicate that subsequent to their presence in early/sorting endosomes the four FGF receptors are sorted differently and that the major part of internalized FGFR1-3 is sorted to lysosomes while the major part of internalized FGFR4 is not.

In attempts to further assess the different sorting of the receptors and to decide the localization of FGF1/FGFR4 complex subsequent to its presence in early/sorting endosomes, the endocytic pathway followed by the fluorescent FGF1 together with the different FGF receptors was compared with the pathways taken by epidermal growth factor (EGF) and transferrin. The most comprehensive studies of tyrosine kinase receptor endocytosis have been carried out using the EGF receptor as an experimental model, demonstrating that the EGFR and its ligand progress to lysosomes upon internalization[9]. On the other hand, the transferrin receptor and its ligand are known to be recycled from early/sorting endosomes via the endosomal recycling compartment back to the cell surface[16].

HeLa cells transfected with the different FGF receptors were incubated for 2 h at 37°C with Alexa 488 labelled EGF and Cy3-labelled FGF in the presence of leupeptin. Cy5-labelled transferrin was added after 90 min. Colocalization was demonstrated in overlay experiments when spots labelled with Cy3-FGF1 and alexa 488 EGF appeared yellow and spots labelled with Cy3-FGF1 and Cy5-transferrin appeared purple. Consistent with previous findings, fluorescent FGF1 endocytosed by FGFR1-3 showed considerable overlap with fluorescent EGF, indicating that the major part of internalized FGF1/FGFR1-3 complexes accumulates in lysosomes. On the other hand, fluorescent FGF1 endocytosed by FGFR4 showed a notable overlap with transferrin,

**Figure 5. Localization of endocytosed EGF, transferrin and FGF1. (A)** HeLa cells were transiently transfected with FGFR1, 2, 3 or 4 and incubated for two hours at 37°C with Cy3-FGF1 and Alexa488-EGF in the presence of 20 U/ml heparin and 0.3 mM leupeptin. Cy5-transferrin (Tf) was added after 90 min. The cells were fixed and examined by confocal microscopy. Arrows point to colocalization or lack of colocalization. Scale bar, 5 μm. **(B)** The percentage of FGF1 positive structures within cells transfected with FGFR1, 2, 3 or 4 that colocalize with EGF, Tf, neither EGF nor Tf or both EGF and Tf was quantified as described in materials and methods. Error bars denote the standard deviation, n=15.


A
FGFR1
FGFR2
FGFR3
FGFR4
FGF1
EGF
Tf
Merge
B
% FGF1 positive structures
80
60
40
20
0
R1
R2
R3
R4
FGF1+EGF
FGF1+TF
FGF1
FGF1+EGF+TF

indicating that a great part of the internalized FGF1/FGFR4 complexes accumulate in the endocytic recycling compartment (Figure 5A).

Quantification of colocalization was performed as described in materials and methods. As shown in Figure 5B approximately 65% of the FGF1 positive structures in cells transfected with FGFR1 colocalized with intracellular structures containing EGF, while only around 8% of the FGF1 positive structures colocalized with intracellular structures containing transferrin. In cells transfected with receptor 2 or 3 between 40 and 50% of the FGF1 positive structures also contained EGF, while about 20% of the FGF1 positive structures contained transferrin. In the case of cells transfected with FGFR4 only around 20% of the FGF1 positive structures contained EGF, while around 50% of the FGF1 positive structures contained transferrin. Noteworthy, between 20 and 30% of the FGF1 positive structures in the transfected cells did not contain EGF nor transferrin and a small fraction of around 5% of the FGF1 positive structures contained both EGF and transferrin.

## Degradation of internalized FGF1 and FGFRs

We then considered the possibility that the different sorting of the four related FGFRs could results in different kinetics of degradation. To study this, the degradation of FGF1 internalized by the four FGF receptors was analysed in FGFR-transfected HeLa cells.

The cells were incubated with radiolabelled 18 kDa form of FGF1 for 30 min to allow endocytosis of the growth factor/receptor complex to occur. The cells were then washed to remove surface-bound FGF1 and further incubated for the indicated periods of time. Finally, the cells were lysed and solubilized proteins were adsorbed to heparin-Sepharose and analysed by SDS-PAGE. The degradation of FGF1 can be seen in Figure 6A as a stepwise conversion of the 18 kDa form of FGF1 into the shorter 16 kDa form followed by further degradation.

Further degradation of FGF1 seems to occur more slowly, indicating that the shorter form of FGF1 is more resistant to degradation than the 18 kDa form of FGF1.

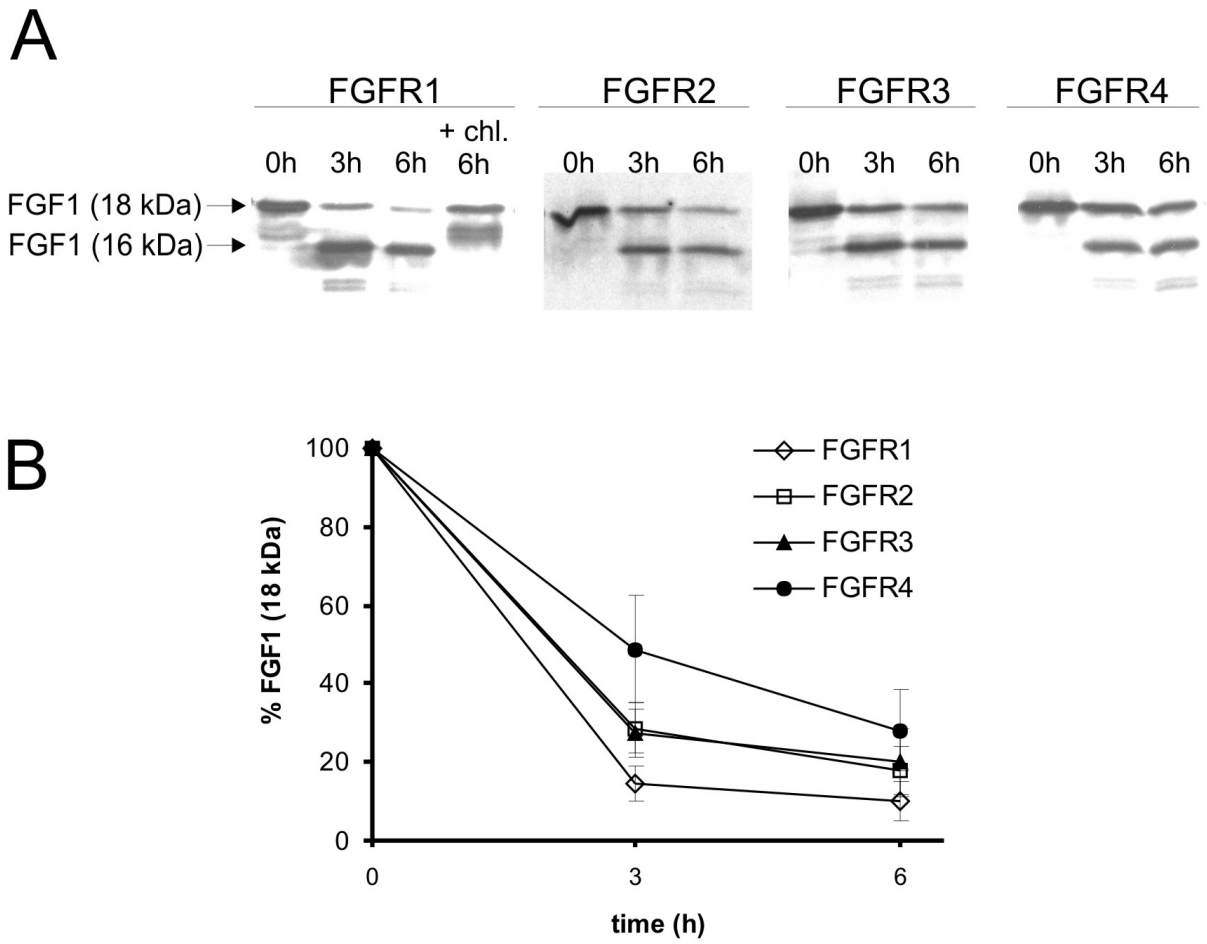



**Figure 6. Degradation of endocytosed long form of FGF1 (18 kDa). (A)** HeLa cells, transiently transfected with FGFR1, 2, 3 or 4 were incubated with [$^{35}$S]methionine-labelled, 18 kDa form of FGF1 and 20U/ml heparin at 37°C for 30 min to allow endocytosis via high affinity receptors. The cells were then either lysed immediately (0h) or incubated further in growth medium with or without chloroquine at 37°C for 3 or 6 hours before lysis. [$^{35}$S]methionine-labelled FGF1 was extracted from the lysate by binding to heparin-Sepharose and analysed by SDS-PAGE. **(B)** For each receptor the amount of the 18 kDa form of FGF1 was calculated at each time point and expressed as a percentage of the amount of the 18 kDa form of FGF1 at time point 0. Values are averages of 5 independent experiments for FGFR1 and FGFR4 and 3 independent experiments for FGFR2 and FGFR3. Error bars denote the standard deviation.

Degradation of internalized FGF1 was inhibited by the weak base chloroquine (shown only for FGFR1), suggesting that the digestion occurred in a lysosomal compartment.

After 6 hours only a small fraction of FGF1 remained as the 18 kDa form in cells transfected with FGFR1, whereas a significant amount of the 18 kDa form of FGF1 was detected in cells transfected with FGFR4. The amount of the 18 kDa form of FGF1 that remained in the cells was calculated for each receptor type and each time point, and expressed as a percentage of the amount of the 18 kDa form of FGF1 at time point 0. The values plotted in the graph in Figure 6B are average values from 5

(for FGFR1 and FGFR4) and 3 (for FGFR2 and FGFR3) independent experiments. Approximately 50% of the FGF1 exist as the 18 kDa form in FGFR4 transfected cells after 3 hours, whereas only 15% of the FGF1 remained as the 18 kDa form after 3 hours in cells transfected with FGFR1. The amount of the 18 kDa form of FGF1 in cells transfected with FGFR2 or FGFR3 was reduced to about 30% after 3 hours. These results show that FGF1 endocytosed via FGFR4 are more slowly degraded than FGF1 endocytosed via FGFR1. FGF1 endocytosed via FGFR2 or 3 seems to be more slowly degraded than FGF1 endocytosed via FGFR1, but faster than FGF1 endocytosed via FGFR4.

To analyse the degradation of internalized receptors, biotinylation of cell-surface proteins was carried out on cells transfected with the different FGF receptors. The cells were then stimulated with FGF1 for indicated periods of time and the biotinylated proteins in the collected lysates were pulled down with streptavidin-Sepharose followed by immunoblotting with appropriate receptor antibodies. Gradual disappearance of the bands in the immunoblots corresponding to the receptors demonstrates degradation of the internalized receptors (Figure 7). The decreased intensity of the bands revealed that internalized FGFR1 was efficiently degraded after 2 hours whereas the amount of internalized FGFR4 was only slightly decreased after 6 hours. The degradation of FGFR2 and FGFR3 seem to occur slower than the degradation of FGFR1 but considerable faster than FGFR4.

The amount of FGFRs that remained in the cells was calculated for each receptor type and each time point, and expressed as a percentage of the amount of receptors at time point 0. The mean values from three independent experiments plotted in the graph in Figure 7B demonstrate a decrease in the amount of FGFR1 from 100 to ~15% in two hours whereas the amount of receptor 4 was decreased to ~80% in six hours. In the case of FGFR2 and FGFR3 30-40% of the receptors were detectable after 6 hours. These findings indicate that FGFR4 are more slowly degraded than FGFR1. FGFR2 and FGFR3 seem to be more slowly degraded than FGFR1, but faster degraded than FGFR4. These data together with the results in Figure 6 support the previous findings

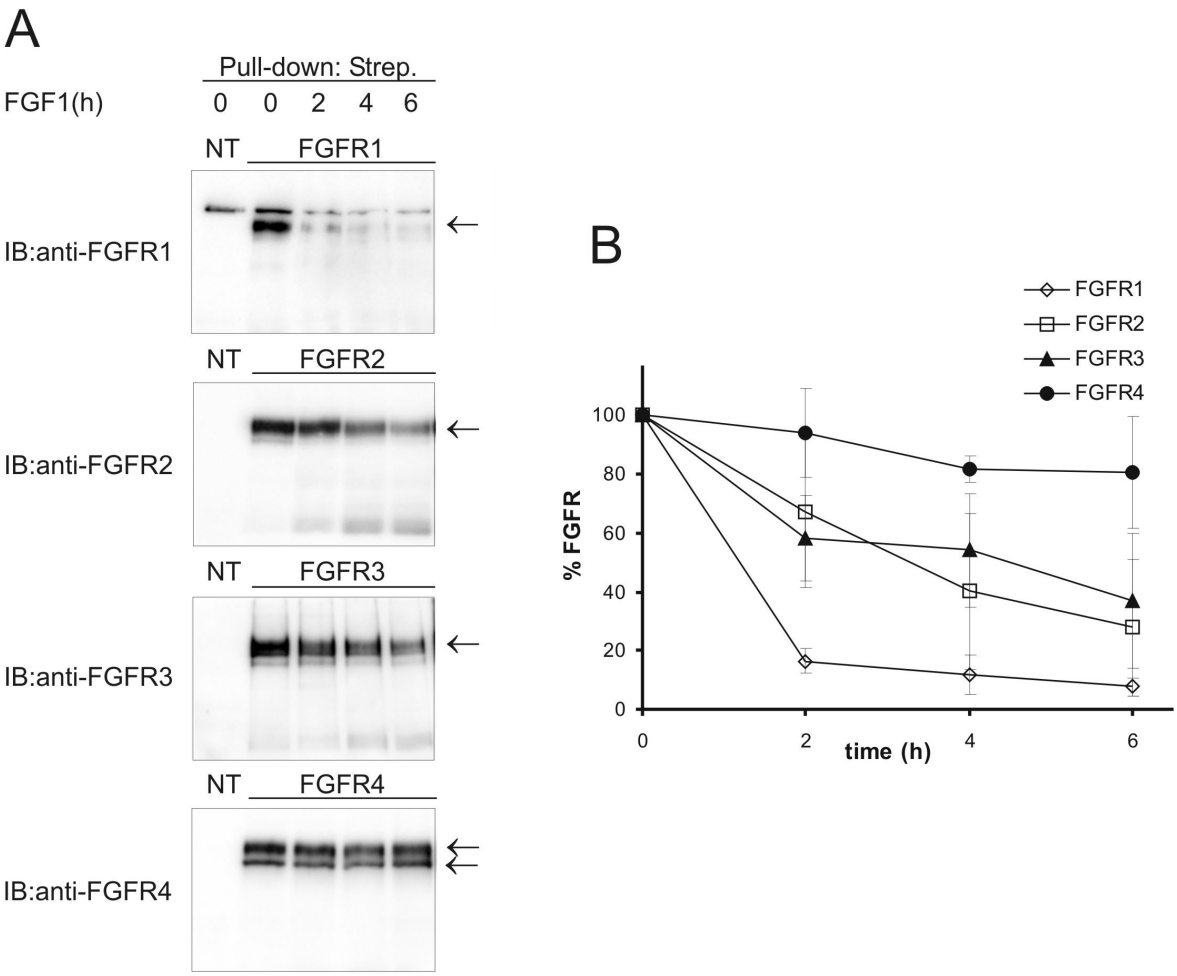



**Figure 7. Degradation of endocytosed FGFRs. (A)** Cell surface proteins on HeLa cells, not transfected (NT) or transiently transfected with FGFR1, 2, 3 or 4 were biotinylated and the cells were incubated for the indicated periods of time at 37°C in the presence of 100 ng/ml FGF1 and 20 U/ml heparin. Biotinylated proteins from lysed cells were adsorbed to streptavidin Sepharose and analysed by immunoblotting (IB) with appropriate anti-FGFR antibodies. **(B)** The intensity of the bands at time point zero was set to 100% and the relative amount of receptors at each time point was measured. Values are averages of three independent experiments. Error bars denote the standard deviation.

that the major part of FGF1 endocytosed together with receptor 4 recycles, whereas the major part of FGF1 internalized by receptor 1, 2 and 3 accumulates in lysosomes.

## Ubiquitination of internalized FGFRs

The attachment of ubiquitin to the intracellular part of a membrane protein is thought to function as a signal for lysosomal degradation[14,33]. If ubiquitination is responsible for the observed different sorting of the FGF receptors, one would expect FGFR4 to become less ubiquitinated than FGFR1. An amino acid sequence alignment of the intracellular part of the four receptors revealed several lysines conserved in FGFR1-3 that were absent in FGFR4 (Figure 8). The intracellular part of receptor 1 and 2


**Figure 8. Amino acid sequence alignment of the intracellular part of FGFR1-4.** The amino acid sequence alignment of the intracellular part of FGFR1, FGFR2, FGFR3 and FGFR4 was created using the Vector NTI 9.0 software based on a Clustal W algorithm[36]. The protein sequences were obtained from the following DNA sequences at NCBI; M34641, BC039243, NM_000142 and X57205. A dash represents a gap introduced to optimize the alignment. Lysines conserved in all the four receptors are indicated in light grey while other lysines are indicated in dark grey.

contain 29 lysines, the intracellular part of FGFR3 contains 25 lysines whereas only 16 lysines were found in the intracellular domain of FGFR4. This finding suggested that the level of ubiquitination might be the reason for the different sorting of the receptors.

To investigate whether internalized FGFR4 is less ubiquitinated than internalized FGFR1, HeLa cells co-expressing the myc-tagged ubiquitin and either FGFR1, FGFR4

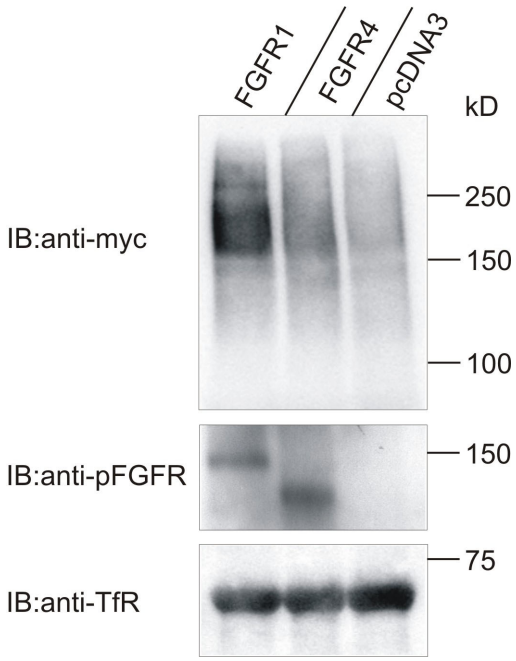



**Figure 9. Ubiquitination of endocytosed FGFRs.** HeLa cells were cotransfected with myc-ubiquitin and FGFR1, FGFR4 or the empty vector (pcDNA3) and starved over night. Cell-surface proteins were biotinylated and the cells were incubated for indicated periods of time at 37°C in the presence of 200 ng/ml FGF1 and 20 U/ml heparin. Biotinylated proteins from lysed cells were adsorbed to streptavidin Sepharose and analysed by immunoblotting (IB) with anti-myc antibodies. The membrane was stripped and reprobed with anti-phospho FGFR antibodies (anti-pFGFR) and anti-transferrin receptor antibodies (anti-TfR).

or an empty vector were starved over night. The starvation was included to avoid stimulation and possibly ubiquitination of other surface proteins by factors in the serum.

Cell-surface proteins were then biotinylated and stimulated with FGF1 for 2 hours. Leupeptin was added to prevent lysosomal degradation of the receptors. The cells were then lysed and biotinylated proteins were pulled down and analysed by western blotting using anti-myc antibody (Figure 9). Ubiquitination of both FGFR1 and FGFR4 was detected as a smear of bands migrating more slowly than the receptors as such. The signal was much stronger for FGFR1 than for FGFR4, indicating that FGFR1 is more ubiquitinated. Very little ubiquitination was detected in cells transfected with the empty vector.

Equal amount of the FGFRs on the membrane was verified by membrane stripping and reprobing with an anti-phospho FGFR antibody that is raised by immunizing rabbits with a synthetic peptide corresponding to residues surrounding tyrosine 653/654 of human FGFR1, which are conserved in FGFR1, 2, 3 and 4. The results indicate that similar amounts of FGFR1 and FGFR4 were analysed (Figure 9). To test for equal loading on the gel, the membrane was stripped and reprobed with anti-transferrin receptor antibodies. The bands were about equally strong in each case (Figure 9).

Equal expression of the myc ubiquitin construct was confirmed by immunofluorescence microscopy (data not shown).

Alltogether, the observations demonstrate that FGFR4 is less ubiquitnated than FGFR1 and they suggest that the different levels of ubiquitination are the molecular mechanism determining their different sorting.

## *Discussion*

In order to compare the intracellular trafficking of FGF1 endocytosed by the four related FGFRs, transiently transfected HeLa cells which do not express detectable amounts of endogenous FGFRs were chosen as a model system. The present work demonstrates that FGF1 internalized by FGFR1, FGFR2 and FGFR3 are targeted for lysosomal degradation whereas the majority of FGF1 internalized by FGFR4 escapes into a recycling pathway. Furthermore, FGF1 endocytosed by FGFR4 was more slowly degraded than FGF1 endocytosed by FGFR1, 2 or 3. Also, FGFR4 itself was more slowly degraded than the other receptors.

Targeting of receptors for lysosomal degradation has been associated with ubiquitination of the intracellular part of the receptors. Consistent with the observed different sorting of the FGFRs, FGFR4 seems to be less ubiquitinated than FGFR1. This indicates that different levels of ubiquitination of the FGFRs might define their intracellular sorting.

The present findings are in accordance with previous data concerning the trafficking of endocytosed FGFRs. KGF/KGFR (FGF7 and a splicing variant of FGFR2) in HeLa cells and FGF2/FGFR3 in RCJ cells has previously been reported to enter the lysosomes upon internalization[1,4] and internalized FGF1/FGFR4 was found to accumulate in the recycling compartment in COS cells[6]. It has also been reported that binding of FGF to FGFR1 in HeLa cells[40] and PAE cells[24] and binding of FGF to FGFR3 in COS-7 cells[4] and 293T cells[23] induces ubiquitination of the receptors and that this contributes to their downregulation. Taken together these findings indicate that the distinct sorting of the FGF receptors is dependent on receptor type rather than cell type.

The juxtanuclear localization of FGF1/FGFR4 previously described in COS cells[5,6], was not observed in HeLa cells, although the major part of the receptors seems to localize to the endocytic recycling compartment in both cases. This could simply be explained by morphological differences between the two cell lines. In some cell types, the endocytic recycling compartment is concentrated near the centriole, whereas the compartment is distributed more widely throughout the cytoplasm in others[21]. The more wide distribution of the endocytic recycling compartment in HeLa cells made it easier to quantify the different trafficking of the receptors by laser scanning confocal microscopy.

Late endosomes and lysosomes contain large amounts of glycoproteins such as lysosomal-associated membrane protein-1 (LAMP-1)[10]. The staining of these compartments for laser scanning confocal microscopy analysis with primary antibodies against LAMP-1 and fluorophore-conjugated secondary antibodies gave a dense pattern of LAMP-1 positive structures. The dense pattern may have caused an overestimation of the degree of colocalization between FGF1 positive structures and LAMP-1 positive structures. In FGFR4 transfected cells approximately 45% of the FGF1 positive structures were positive for LAMP-1. However, when the distribution of fluorophore-labelled FGF1 internalized via FGFR4 was compared with the distribution of internalized fluorophore-labelled EGF, a marker for lysosomal trafficking, only 20% of the FGF1 positive structures contained EGF.

When continuous uptake of fluorophore-labelled FGF1 was allowed in cells transfected with the different receptors, between 20 and 30% of the FGF1 positive structures inside the cells colocalized neither with the marker for lysosomal trafficking (EGF) nor the marker for the recycling pathway, transferrin. It is likely that the amount of overexpressed FGFRs exceeds the amount of the other receptors at the cell surface. Therefore, free FGFRs could still be present at the cell surface, ready to bind ligand and internalize when most of the EGF and transferrin receptors are already located in intracellular vesicles. Some of the FGF1 positive structures inside the cells also contained both fluorophore labelled EGF and transferrin. Since EGF and transferrin are known to enter early endosomes upon internalization, it is not surprising that some

intracellular vesicles contained both ligands when continuous uptake of ligands was allowed.

The degradation of internalized FGF1 was seen as a stepwise conversion of the 18 kDa form of FGF1 into a shorter 16 kDa form. Further degradation of the short form of FGF1 seemed to occur more slowly, indicating that the shorter form of FGF1 is more resistant to degradation than the long form of FGF1. Internalized FGF2 in BCE cells have been reported to be rapidly cleaved from an 18 kDa form to a 16 kDa form and the 16 kDa form was then found to be more slowly degraded with a half-life of approximately 8 hours[25]. This seems to be the case for FGF1 as well.

The kinetics of FGFR degradation has been addressed in a few studies[1,4,35]. Common for these studies are metabolical labelling of cells, followed by immunoprecipitation of the FGFRs and analysis of the relative amount of the remaining FGFRs by autoradiography. This approach was not suitable for our purposes as a considerable amount of overexpressed proteins seems to be degraded at the endoplasmic reticulum (ER). In a metabolic labelling pulse chase experiment recently reported[34], about 60-80% of the FGFR4 was degraded after 4 hours when cells overexpressing the receptor were treated with brefeldin A to inhibit transport out of the ER. Even though the degradation in the ER probably was increased as the transport out of the ER was blocked, this experiment indicates that a considerable amount of the overexpressed receptors is degraded in the ER. In our report the detection of lysosomal receptor degradation was ensured by the extraction of biotinylated cell-surface proteins. Ligand independent degradation of metabolically labelled FGFR1 and FGFR2 has also been reported[1,35]. This could be explained partly by ER degradation due to receptor overexpression, but not totally as the cells used in the case of FGFR1 were stably transfected. It is therefore not excluded that a constitutive turnover of the receptors from the cell surface takes place.

All the four FGFRs have been found to have distinct patterns of distribution in many human tissues. The most widespread expression has been observed for FGFR1 whereas FGFR4 was found to have a more limited distribution[17]. FGFR mouse gene

knockout and mutational studies have implicated FGFR1-3 in numerous developmental events while FGFR4 seems to play a more modest role in developmental processes[12,28,41]. It is possible that the limited function in developmental events and the restricted distribution of FGFR4 could be explained by the different sorting of the FGFRs. The recycling of FGFR4 might prolong its signalling activities and the signalling might be further prolonged if the ligands internalized by FGFR4 are allowed to reappear at the cell surface and activate new FGFRs. It is therefore likely that FGFR4 is less involved in processes where accurate downregulation of signalling receptors is necessary. However, the recycling of FGFR4 may on the other hand provide a mechanism for gradient formation during developmental processes. A simple model of gradient formation postulates that morphogens dilute as they diffuse between cells. Recent data supports the idea that movement of morphogens could also occur by vesicular trafficking through the cells[8]. Recycled morphogens can thus be re-secreted and move forward into the target tissue. FGFs could therefore after binding and activation of FGFR4 in one cell, be recycled and activate neighbouring cells and spread through the tissue.

FGFRs have been found overexpressed or mutated to constitutively active forms in several human cancers[30]. It might be suggested that elevated levels or constitutively active forms of FGFR4, to a greater extent than the other FGFRs, predisposes cancer patients for accelerated disease progression because they are not efficiently downregulated. On the other hand, examination of constitutively activated derivatives of FGFR1, FGFR3 and FGFR4 in which a myristylation signal was substituted in place of the extracellular and transmembrane domains, thereby targeting the kinase domain to the plasma membrane, revealed that FGFR4 was much less transforming than activated FGFR1 and FGFR3[15]. Since FGFR1 also exhibit higher signalling activity than FGFR4 it has been suggested that FGFR1 is the most potent mutagenic member of the FGFR family[37,38]. Other mechanisms for attenuating signals may therefore play a role to limit the signalling from the FGFR4 that is less efficiently degraded.

Although the exact biological role of the different trafficking of the FGF receptors remains to be revealed, further studies on the basic biology of FGFs, FGFRs and their signalling is of importance. Knowledge about how the growth factors and their corresponding receptors function is crucial in order to try to stimulate or inhibit their effects for possible therapeutic purposes.

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