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PROMOTION OF NEURITE OUTGROWTH BY FIBROBLAST GROWTH FACTOR RECEPTOR 1 OVEREXPRESSION AND LYSOSOMAL INHIBITION OF RECEPTOR DEGRADATION IN PHEOCHROMOCYTOMA CELLS AND ADULT SENSORY NEURONS

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Abstract—Basic fibroblast growth factor (FGF-2) is up-regulated in response to a nerve lesion and promotes axonal regeneration by activation of the tyrosine kinase receptor fibroblast growth factor receptor 1 (FGFR1). To determine the effects of elevated FGFR1 levels on neurite outgrowth, overexpression was combined with lysosomal inhibition of receptor degradation. In pheochromocytoma (PC12) cells, FGFR1 overexpression resulted in flattened morphology, increased neurite outgrowth and activation of extracellular signal-regulated kinase (ERK) and AKT. Degradation of FGFR1 was inhibited by the lysosomal inhibitor leupeptin and by the proteasomal inhibitor lactacystin. In rat primary adult neurons, FGFR1 overexpression enhanced FGF-2-induced axon growth which was further increased by co-treatment with leupeptin. Lysosomal inhibition of receptor degradation concomitant with ligand stimulation of neurons overexpressing FGFR1 provides new insight in tyrosine kinase receptor–mediated promotion of axon regeneration and demonstrates that adult sensory neurons express sub-optimal levels of tyrosine kinase receptors for neurotrophic factors.

Key words: FGFR1, MAP kinase, leupeptin, lysosome, sensory neurons, axonal growth.

A subset of the highly conserved fibroblast growth factors (FGFs) (Ornitz and Itoh, 2001) belongs to the neurotrophic factor family (Baird, 1994). FGF-2 (basic FGF) plays a prominent role in axonal growth not only during nervous system development (Mason, 2007), but also as a maintenance factor after brain injury or after nerve lesion in the adult nervous system (Reuss and von Bohlen und Halbach, 2003; Grothe et al., 2006). FGF-2 is up-regulated in response to nerve injury in peripheral ganglia and in the lesioned nerve (Grothe and Nikkhah, 2001). Within ganglia FGF-2 is expressed by neurons and glial cells, whereas at the lesion site Schwann cells and macrophages represent the main sources of FGF-2 (Ji et al., 1995; Grothe et al., 1997; Klimaschewski et al., 1999). Exogenously applied FGF-2 promotes neuronal survival and neurite outgrowth *in vitro* and *in vivo* (Grothe et al., 2006). In the sciatic nerve lesion model, axotomized dorsal root ganglion (DRG) neurons are rescued by FGF-2 (Otto et al., 1987) and regeneration of lesioned axons is enhanced across FGF-2-filled nerve conduits (Danielsen et al., 1988; Aebischer et al., 1989). Moreover, Schwann cells overexpressing FGF-2 stimulate axonal regrowth across long gaps (Haastert et al., 2006). FGF-2 isoforms significantly enhance elongating axon growth by adult sensory neurons in response to a preconditioning sciatic nerve lesion (Klimaschewski et al., 2004).

FGFs mediate their response by activation of four types of high affinity tyrosine kinase receptors (FGFR1-4). FGFR1-4 participate in neuronal FGF signaling with FGFR1 being the most abundant fibroblast growth factor receptor (FGFR) in the nervous system (Ford-Perriss et al., 2001). FGFR1, -2 and -4 are expressed in the rodent DRG albeit at different levels, while FGFR3 is up-regulated in response to a nerve lesion (Oellig et al., 1995; Grothe et al., 1997, 2001). Characteristic for FGFR1-3 is the occurrence of numerous receptor isoforms that are produced from alternative mRNA splicing (Itoh and Ornitz, 2004), which regulates the number of immunoglobulin-like (Ig-like) domains (two or three) and the specific sequence of Ig domain III (Johnson et al., 1990, 1991). The original FGFR1 cloned from chicken contains three extracellular Ig-like domains and is termed 'fibroblast growth factor receptor 1 long' (FGFR1α or FGFR1$_L$). In contrast, 'fibroblast growth factor receptor 1 short' (FGFR1β or FGFR1$_S$) contains two extracellular Ig-like loops only. The expression of the different isoforms is tissue-specific and, in the brain, the long form represents the major isoform (Yazaki et al., 1993). FGFR1$_S$ displays a 10-fold higher affinity to both, FGF and heparin, compared with FGFR1$_L$ (Bernard et al., 1991; Wang et al., 1995; Groth and Lardelli, 2002). Although it seems possible that the additional Ig-like loop of the FGFR1$_L$ displays an autoinhibitory function (Plotnikov et al., 1999), which prevents FGF-independent activa-

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Abbreviations: DRG, dorsal root ganglia; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FGFR1$_L$, fibroblast growth factor receptor 1 long; FGFR1$_S$, fibroblast growth factor receptor 1 short; Ig-like, immunoglobulin-like; MAP, mitogen-activated protein; MD, maximal distance; NGF, nerve growth factor; PC12, pheochromocytoma; TrkA, tropomyosin-related kinase A.

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tion by heparin sulfate proteoglycans, $FGFR1_S$ and $FGFR1_L$ exhibit the same strong responses to FGF-2 and the physiological significance of inclusion or exclusion of the Ig-like loop is still largely unknown. By contrast, the ligand-binding specificity of alternatively spliced Ig-like domain III (the IIIb and IIIc isoforms) is different. FGF-2 exhibits a higher affinity to FGFR1c than to FGFR1b (Ornitz et al., 1996).

Ligand binding in cooperation with the accessory heparin sulfate proteoglycans leads to FGFR dimerization and autophosphorylation of their cytoplasmic domains, which recruits a number of signaling molecules relevant for axonal growth (Mason, 2007). The docking protein FRS2 becomes tyrosine-phosphorylated upon FGF stimulation and associates with the Grb/SOS complex to relay activation of the downstream mitogen-activated protein (MAP) kinase pathway (Kouhara et al., 1997). Extracellular signal-regulated kinase (ERK) activation by FGFR leads to a sustained and robust signal that stimulates neuronal differentiation (Traverse et al., 1992). Activation of ERK/MAP kinases seems to be the common response mediated by all FGFRs, whereas p38 or AKT/protein kinase B activation may be cell type specific (Dailey et al., 2005). FGFR activation is followed by rapid endocytosis and degradation of both, the receptor and the ligand, accompanied by the termination of the signal. Multiple mono-ubiquitination of the receptor by the ubiquitin ligase c-Cbl, via interaction of FRS2 and Grb2, leads to sorting into intraluminal vesicles of multivesicular endosomes and subsequent delivery to lysosomes followed by degradation via lysosomal enzymes (Wong et al., 2002; Bache et al., 2004). Among all four FGFRs, FGFR1 revealed the highest levels of ubiquitination and the fastest lysosomal degradation (Haugsten et al., 2005).

In this study, we investigated the effects of increased FGFR1 levels on FGF-2 induced neurite outgrowth by rat pheochromocytoma (PC12) cells and adult sensory neurons from DRG, and analyzed the effects of leupeptin, an inhibitor of lysosomal proteases. PC12 cells provide a well-characterized model of neurite outgrowth (Greene and Tischler, 1982). They differentiate morphologically and biochemically into peripheral neuron-like cells when treated with neurotrophic factors like nerve growth factor (NGF) (Togari et al., 1985; Rydel and Greene, 1987). The data presented here demonstrate that FGFR1 overexpression promotes neurite outgrowth by PC12 cells and DRG neurons, and that this effect can be significantly enhanced by the lysosomal inhibitor leupeptin, suggesting that the sensitivity of adult peripheral neurons to growth factor treatment may be increased by elevated growth factor receptor levels.

EXPERIMENTAL PROCEDURES

Cell culture and transfection

PC12 cells were cultured in collagen-coated plastic dishes or matrigel™-coated (BD Biosciences, Vienna, Austria) glass dishes under standard conditions in a humidified atmosphere at 37 °C with 5% CO_2. RPMI 1640 cell culture medium (Gibco Invitrogen, Lofer, Austria) supplemented with 10% horse serum (Gibco Invitrogen), 5% fetal bovine serum (Gibco Invitrogen) and antibiotic-antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, Gibco Invitrogen) was changed two times a week. $FGFR1_S$ (FGFR1β, two Ig-like domains, IgIIIc) and $FGFR1_L$ (FGFR1α, three Ig-like domains, IgIIIc) fused to EGFP (gifts from Dr. P. Cattini; the study was supported by the Medical University Innsbruck, Christoph-Probst-Platz Innrain 52, A-6020 Innsbruck; Jin et al., 1994) were applied for transfection. PC12 cells were transfected using the Amaxa Nucleofector™ (program U-29). Dissociated PC12 cells (2×10^6) were electroporated with 3 μg of $FGFR1_S$-EGFP, $FGFR1_L$-EGFP or EGFP control plasmids in 100 μl RPMI medium. Clones stably overexpressing $FGFR1_S$ (S1–S4) or $FGFR1_L$ (L1–L4) were selected with G418 antibiotic (Geneticin, Gibco Invitrogen).

The experiments presented here are in accordance with the statement of ethical standards concerning animal care of the guidelines of the National Institutes of Health and the ethical commission of the Austrian Ministry of Science. The number of animals was kept to a minimal degree and any suffering was strictly avoided. Adult rat DRG were dissected, collected in RPMI medium with 1% antibiotic–antimycotic and treated with collagenase (5000 units/ml) for 60 min followed by 0.25% trypsin/EDTA for another 15 min. They were then transferred to RPMI medium containing 10% horse serum and 5% fetal bovine serum and dissociated by 5 to 15 passages through a fire-polished Pasteur pipette. Ten softened DRGs were electroporated with 5 μg of $FGFR1_S$-EGFP, $FGFR1_L$-EGFP or EGFP control plasmids in 100 μl Rat Neuron Nucleofector® solution using the Amaxa Nucleofector™ (program O-O3) and plated on glass dishes coated with 0.1 mg/ml poly-D-lysine (overnight) and 0.2 mg/ml laminin (for 4 h). Cultures were maintained in RPMI medium with B27 supplement (Gibco Invitrogen) and antibiotic–antimycotic at 37 °C in a humidified atmosphere with 5% CO_2. Medium was changed carefully every day to remove cellular debris. Transfected cells were documented using an inverted fluorescence microscope (Zeiss Axiovert 100) equipped with a SPOT RT digital camera connected to a PC.

Measurement of receptor fluorescence and neurite outgrowth

PC12 cells and neurons overexpressing EGFP-fused $FGFR1_S$, $FGFR1_L$ or EGFP alone were treated with NGF (100 ng/ml, Sigma, Vienna, Austria), FGF-2 (100 ng/ml plus heparan sulfate 10 μg/ml, Sigma), the proteasome inhibitor lactacystin (5 μM, Sigma) or the lysosome inhibitor leupeptin (0.3 mM, Sigma) diluted in RPMI medium with N2-supplement (Gibco Invitrogen) for PC12 cells or B27-supplement (Gibco Invitrogen) for neurons. One day after transfection, whole cell fluorescence intensities correlating with receptor levels were observed every 10 min for 1 h after addition of FGF-2 (plus heparan sulfate). PC12 cells were pre-incubated with lactacystin (for 5 h) or with leupeptin (for 2 h).

Average cell fluorescence intensities of transiently transfected PC12 cells were measured after background subtraction applying Metamorph software (MetaMorph®, version 4.6, Visitron Systems). For measurement of neurite outgrowth, four PC12 cell clones stably overexpressing EGFP-fused $FGFR1_S$ (S1–S4) or $FGFR1_L$ (L1–L4) were selected. MetaMorph® morphometry software was applied to measure the longest of all vectors from the center of the cell body to the growth cones (maximal distance, MD) in PC12 cell cultures (4 days after growth factor addition) and the total axonal length and the MD of primary DRG neurons (1 day after growth factor addition). Neurons overexpressing $FGFR1_S$ or $FGFR1_L$ were identified by EGFP fluorescence and documented at days 2 and 3 after transfection followed by image correction for background and cell body fluorescence. All morphologically intact neurons per dish with MDs $\geq$50 μm were analyzed and the same cells were documented on day 2 and day 3 (recovered by x- and y-coordinates).

Western blotting

Cells were homogenized in lysis buffer (50 mM Tris–HCl pH 7.4, 500 mM NaCl, 1% NP-40, 0.5% Na-desoxycholate, 0.1% SDS, 0.05% NaN_3) supplemented with 20 μg/ml complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail I and II (1:100, Sigma). DNA was destroyed by sonication of lysates and the insoluble fraction was removed by centrifugation for 15 min at 4 °C at 10,000×*g*. Protein levels were determined using Bradford protein assay reagent (Biorad, Munich, Germany) and aliquots containing 50 μg of protein were analyzed by electrophoresis on SDS–polyacrylamide gels and then transferred to polyvinylidine difluoride membranes. Primary antibodies against pERK (Cell Signaling, New England Biolabs, Frankfurt am Main, Germany), ERK (Upstate, Lake Placid, NY), pp38 (Upstate) and p38 (Santa Cruz, Santa Cruz, CA), pAKT (Cell Signaling) and AKT (Cell Signaling) were diluted at 1:1000. Secondary horseradish peroxidase–linked anti-rabbit (Pierce, Vienna, Austria) or anti-goat (Jackson Immuno Research, Suffolk, UK) antibody (both 1:10,000) was detected by the enhanced chemiluminescence Western blotting detection system (Pierce).

Quantitative PCR

RNA was isolated with RNeasy Mini Kit (Qiagen, Vienna, Austria) including DNase-digestion. One microgram of RNA was applied to the iScript™ cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions with 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C resulting in 20 μl of cDNA. Real-time PCR was performed on Biorad's iCycler in a final volume of 25 μl with 12.5 μl iQ SYBR Green Supermix (containing 40 mM Tris–HCl, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM of each dNTP, 50 units/mL iTaq DNA polymerase, SYBR Green I, 20 nM fluorescein and stabilizers), 7.5 μl sterile water, 2 μl of each primer (160 nM) and 1 μl cDNA template (3 min denaturing step followed by 40 cycles 30 s at 95 °C and 60 s at 60 °C). Control primers include actin sense 5′dGAAAGGGTGTAAAACGCAGC, antisense 5′dCGATCCGTAAAGACCTCTATGC and HRPT1 sense 5′dTGACACTGGCAAAACAATGCA, antisense 5′dGGTCCTTTTCACCAGCAAGCT. Primers for FGFR1 were sense 5′dGTATGACCAGATCCTGAAGACTGC, antisense 5′dCACCATACAGGAGATCAGGAAGGC; for FGFR1 fused to EGFP were sense 5′dACCGCTGGACCAGTACTCAC, antisense 5′dGGACACGCTGAACTTGTGG. The verification of correct PCR products was performed with both melt curve analysis (Biorad iCycler iQ software) and agarose electrophoresis separation.

Quantitative single cell PCR

Neurons overexpressing EGFP-fused $FGFR1_S$, $FGFR1_L$ or EGFP alone were identified by fluorescence. For cell harvest micropipettes were made by pulling glass capillaries (outer diameter=1 mm, inner diameter=0.58 mm; World Precision Instruments, Berlin, Germany) to an aperture diameter of about 16 μm using a Narishige PP-830 pipette puller. Under visual control the tip of the pipette was gently attached to the selected cells and through a mouthpiece connected to the pipette suction was applied until the cell entered the tip of the pipette. After aspiration of 10 cells at a time, the collected cells were ejected into a tube and immediately homogenized in 20 μl of lysis buffer (Dynabeads mRNA DIRECT™ Micro Kit, Dynal Invitrogen, Lofer, Austria). The RNA was captured by 10 μl Dynabeads Oligo $(dT)_{25}$ and the beads were washed according to the manufacturer's instructions. cDNA synthesis was directly performed with RNA bound beads in a volume of 20 μl iScript™ cDNA Synthesis Kit (Biorad) for 5 min at 25 °C and 30 min at 42 °C, during which beads were kept in suspension by shaking. Afterward the cDNA reaction mix was removed and the beads were resuspended in 10 μl of sterile water. Real-time PCR was performed on Biorad's iCycler in a final volume of 25 μl with 12.5 μl iQ SYBR Green Supermix, 3.5 μl sterile water, 2 μl of each primer (160 nM) and 5 μl cDNA coupled beads (3 min denaturing step followed by 40 cycles 30 s at 95 °C and 60 s at 60 °C). PCR products were verified with both melt curve analysis (Biorad iCycler iQ software) and agarose electrophoresis separation.

RESULTS

Ligand induced FGFR1 degradation is inhibited by leupeptin and lactacystin in PC12 cells

FGF-2-induced degradation of FGFR1 was detected via fluorescence measurements of PC12 cells transiently transfected with $FGFR1_L$-EGFP or $FGFR1_S$-EGFP (Fig. 1). Average fluorescent intensity was measured after background correction from 10 to 60 min after FGF-2 addition. Untreated cells overexpressing $FGFR1_L$-EGFP and $FGFR1_S$-EGFP served as controls for the reduction of fluorescence caused by other factors than FGF-2. PC12 cells transfected with EGFP alone and treated with FGF-2 in the same manner as FGFR1-EGFP transfected cells served as bleaching controls. After FGF-2 treatment, the $FGFR1_L$-EGFP and $FGFR1_S$-EGFP overexpressing cells exhibited a statistically significant reduction in fluorescence as compared with the bleaching controls. The decline in fluorescence without FGF-2 treatment or in EGFP transfected cells (bleaching controls) was significantly weaker than the decline caused by FGF-2 in cells overexpressing $FGFR1_L$-EGFP or $FGFR1_S$-EGFP. Ligand-induced FGFR1 degradation was blocked by pre-incubation with the lysosomal inhibitor leupeptin (0.3 mM, 2 h). Fluorescence levels remained constant after FGF-2 treatment indicating that FGFR1 degradation is impeded by the lysosomal inhibitor. The proteasomal inhibitor lactacystin (5 μM, 5 h) blocked the decrease in receptor fluorescence as well but to a lesser extent than leupeptin.

PC12 cells stably overexpressing FGFR1

PC12 cells used in different laboratories may differ in their response to FGF treatment (Togari et al., 1985; Rydel and Greene, 1987). Only about 1% of the PC12 cells present in our laboratory generated short neurites after 4-day treatment with 100 ng/ml FGF-2 (plus 10 μg/ml heparan sulfate), whereas treatment with NGF (100 ng/ml) resulted in 19% of cells with neurites. Priming with NGF (pretreatment with 100 ng/ml NGF for 4 days, followed by mechanical disruption and re-plating of the cells) resulted in threefold FGFR1 mRNA upregulation. The previously unsusceptible cells generated processes in response to FGF-2 treatment (data not shown), suggesting a crosstalk between FGFR1 and tropomyosin-related kinase A (TrkA) (NGF receptor) signaling and a dependency of FGF-2-induced neurite outgrowth on FGFR1 levels. After transfection with either FGFR1 splice variants fused to EGFP, four PC12 clones stably overexpressing $FGFR1_L$-EGFP (L1–L4) and $FGFR1_S$-EGFP (S1–S4), respectively, were selected with G418 and characterized with regard to their morphological and biochemical properties. PC12 cells stably overexpressing FGFR1 displayed a morphology differ-

ent from naive PC12 cells (Fig. 2). A subpopulation of 3% to 31% of cells, depending on the clone, revealed a characteristic flattened, spindle-shaped morphology with two short cytoplasmic extensions which did not exceed the length of two cell body diameters. This is regarded as the first sign of PC12 cell differentiation prior to neurite outgrowth within minutes to hours after NGF treatment (Rydel and Greene, 1987). In comparison, naive PC12 cells are round to ovoid.

Overexpression of FGFR1 mRNA was confirmed by quantitative real-time PCR (Fig. 3). Measurement of FGFR1 mRNA levels revealed a 250- to a 900-fold increase in $FGFR1_{L1–L4}$ and $FGFR1_{S1–S4}$ clones compared with naive PC12 cells.

Neurite outgrowth by naive and FGFR1 overexpressing PC12 cells in response to FGF-2 and NGF treatment

The percentage of cells with neurites longer than two cell body diameters was determined after 4-day treatment with FGF-2 (100 ng/ml+10 μg/ml heparan sulfate), NGF (100 ng/ml) or FGF-2 plus NGF (Fig. 4). Without growth factor treatment neither naive nor FGFR1 overexpressing PC12 cells generated processes longer than two cell bodies. In contrast, PC12 cells stably overexpressing $FGFR1_L$ (L1) or $FGFR1_S$ (S4) exhibited extensive neurite outgrowth after treatment with FGF-2 compared with naive PC12 cells (similar results obtained with clones L2–L4 and S1–S3, data not shown). In naive PC12 cells, only 1% of the cells formed neurites after 4-day treatment with FGF-2 and treatment for longer than 4 days did not increase the number of cells with neurites. The percentage of cells exhibiting neurites increased up to 20% (L1) in response to FGFR1 overexpression. The potential for FGF-2-induced neurite outgrowth was reflected by the fraction of cells with flattened morphology. The clones L1, L3, S2 and S4, which contain the highest proportion of the flattened, spindle-shaped cells (Fig. 2a), revealed the most prominent neurite outgrowth in the presence of FGF-2. Treatment with NGF resulted in more cells with neurites than with FGF-2 treatment in naive cultures but not in FGFR1 overexpressing clones L1 and S4. Co-treatment with both, FGF-2 and NGF, resulted in more cells with neurites than treatment with FGF-2 or NGF alone in FGFR1 overexpressing but not in naive PC12 cells. The proportion of cells bearing neurites almost doubled in FGFR1 overexpressing cultures in response to co-treatment with FGF-2 and NGF compared with single treatment.

Neurite length was determined by measurement of the MD from the cell body to the longest neurite after 4-day treatment with FGF-2, NGF or FGF-2 plus NGF (Fig. 5). After FGF-2 treatment, the neurite length of $FGFR1_L$ and $FGFR1_S$ overexpressing PC12 cells L1 and S4 doubled when compared with naive PC12 cells. In addition, all FGFR1 overexpressing cells formed longer neurites after FGF-2 treatment than with NGF. Co-treatment with FGF-2 and NGF had no additional effect on the neurite length.

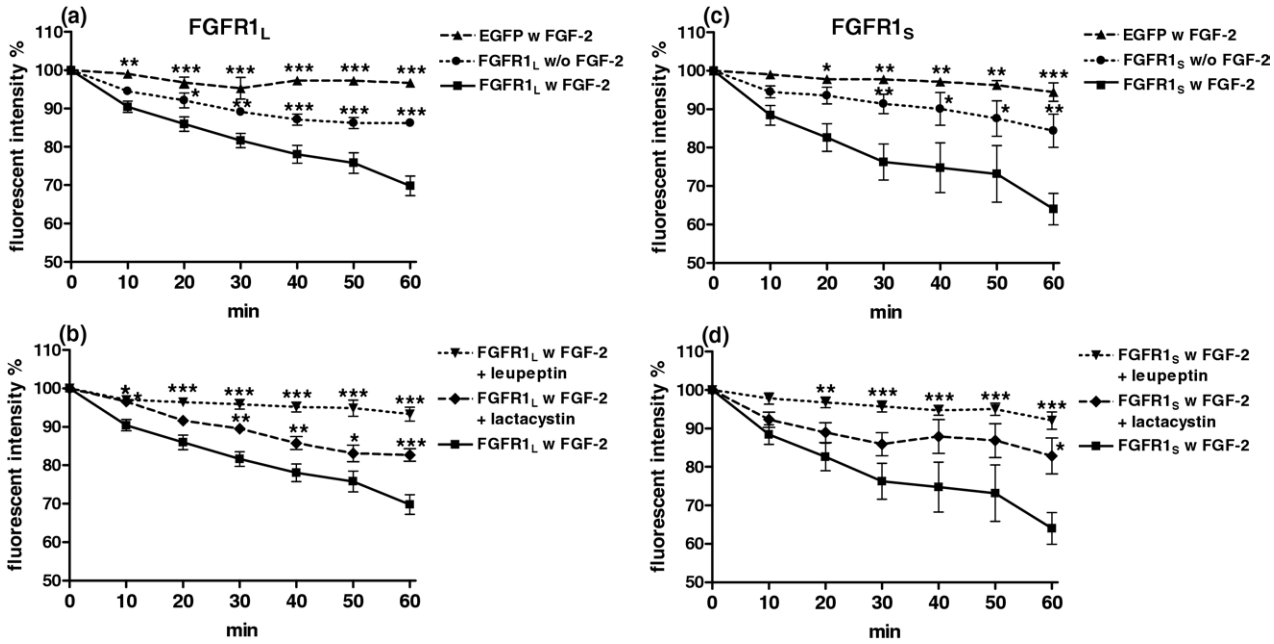

Fig. 1. FGFR1 degradation as detected by fluorescence measurement of PC12 cells transfected with EGFP-fused $FGFR1_L$ (a, b) or $FGFR1_S$ (c, d). Background-corrected average fluorescence intensities were measured for 60 min after growth factor addition (100 ng/ml FGF-2+10 μg/ml heparan sulfate). Untreated (w/o FGF-2) $FGFR1_L$-EGFP or $FGFR1_S$-EGFP transfected cells and EGFP control transfected cells (a, c) treated with FGF-2 served as controls. Inhibition of FGFR1 degradation by pre-incubation of $FGFR1_L$-EGFP (b) or $FGFR1_S$-EGFP (d) transfected cells with the lysosomal inhibitor leupeptin (2 h; 0.3 mM) or with the proteasomal inhibitor lactacystin (5 h; 5 μM) (asterisks indicate significant differences compared with FGF-2-treatment at different times, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, two-way ANOVA with Bonferroni's post test, mean±S.E.M. of four independent experiments with a total number of cells $n>100$).

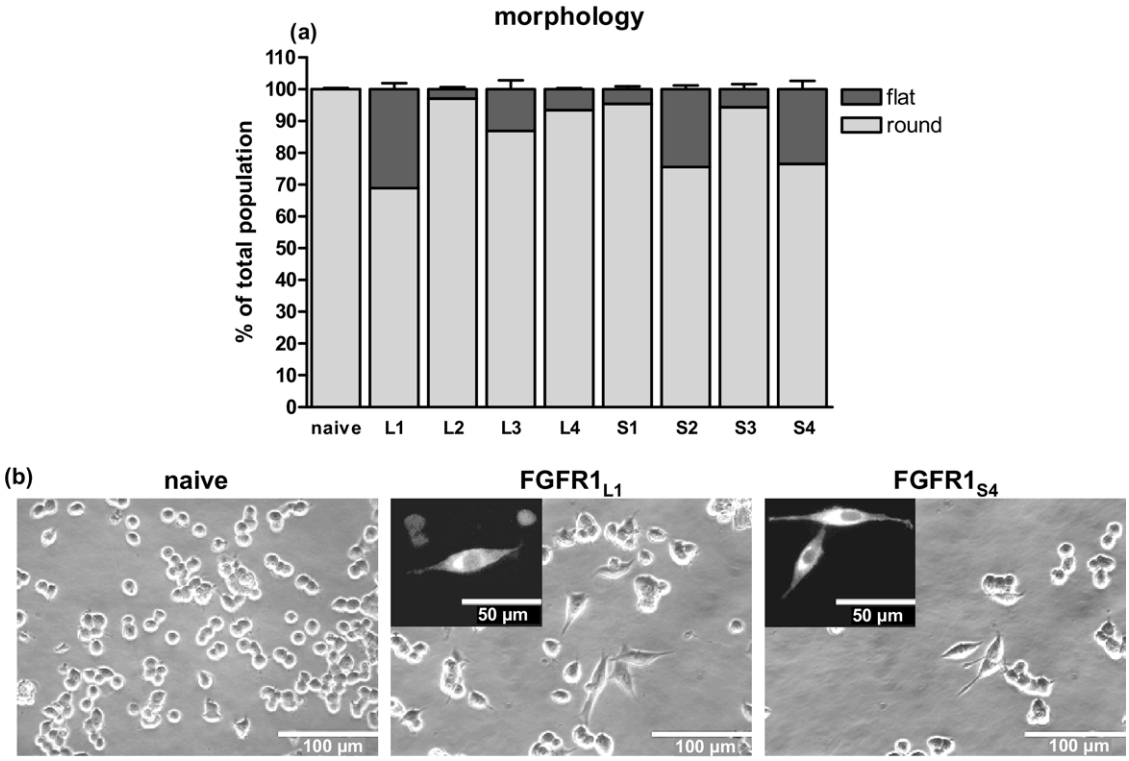

Fig. 2. Different morphology of $FGFR1_L$ (L1–L4) and $FGFR1_S$ (S1–S4) overexpressing PC12 cell clones as compared with naive PC12 cells (mean±S.E.M. of four independent experiments with a total number of cells $n>500$). (a) Percentage of cells with flat or round morphology. (b) Naive cells are round to ovoid, $FGFR1_{L1}$ and $FGFR1_{S4}$ clones exhibit in part a flattened, spindle-shaped morphology.

Leupeptin treatment did not induce neurite outgrowth by $FGFRI_L$ or $FGFR1_S$ overexpressing PC12 cells and had no additional effect on FGF-2-induced neuritogenesis concerning the relative number of neurites and the neurite length (data not shown).

Signaling mechanisms: Kinase activation in FGFR-overexpressing PC12 cells

To explore the signaling pathways involved in FGF-2-induced neurite outgrowth by $FGFR1_L$ and $FGFR1_S$ overexpressing clones L1 and S4, phosphorylation of the kinases ERK, p38 and AKT was analyzed by Western blotting (Fig. 6). Band intensities were quantified using Quantity One (Biorad) software and the ratio phosphoprotein/total protein was calculated. Phosphorylation of ERK was strongly increased 15 min to 2 h after FGF-2 treatment in FGFR1 overexpressing PC12 (L1, S4) cells compared with naive PC12 cells and disappeared again after 4 days. AKT phosphorylation was increased as well, but to a minor degree compared with ERK. The activation of AKT was

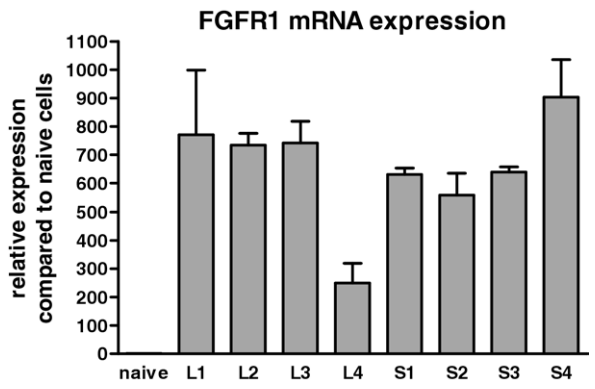

Fig. 3. FGFR1 overexpression by PC12 cell clones. mRNA expression of $FGFR1_L$ (L1–L4) and $FGFR1_S$ (S1–S4) determined by quantitative real-time PCR compared with naive PC12 cells (mean±S.E.M., $n>4$).

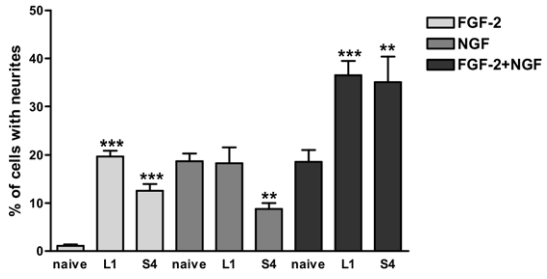

Fig. 4. Neurite outgrowth by FGFR1 overexpressing PC12 cells. Naive PC12 cells and PC12 clones with stable overexpression of $FGFR1_L$ (L1) and $FGFR1_S$ (S4). Bars indicate the proportion of cells with neurites longer than two cell bodies after 4-day treatment with FGF-2 (100 ng/ml+10 µg/ml heparan sulfate), NGF (100 ng/ml) or FGF-2 plus NGF (asterisks indicate significant differences compared with naive cells, ** $P<0.01$, *** $P<0.001$, one-way ANOVA with Tukey's post test, mean±S.E.M. of four independent experiments with a total number of cells $n>1000$).

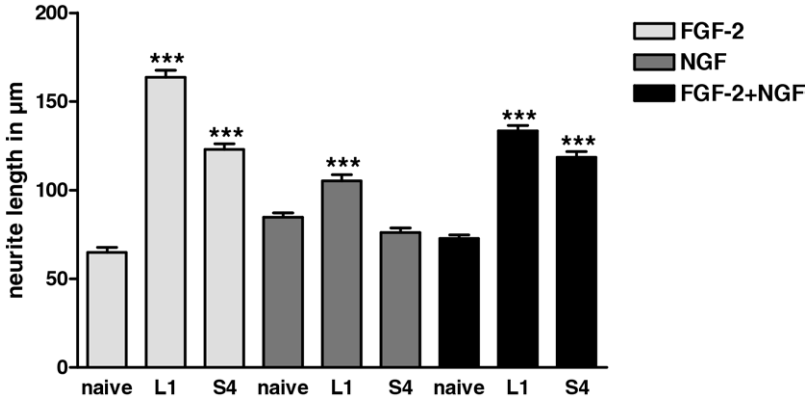

Fig. 5. Mean maximal distance (MD) of FGFR1 overexpressing PC12 cells. Naive PC12 cells and PC12 clones with stable overexpression of $FGFR1_L$ (L1) and $FGFR1_S$ (S4). Bars indicate the MD from the cell body to the longest neurite after treatment with FGF-2 (100 ng/ml+10 μg/ml heparan sulfate), NGF (100 ng/ml) or FGF-2 plus NGF (asterisks indicate significant differences compared with naive cells, *** $P<0.001$, one-way ANOVA with Tukey's post test, mean±S.E.M. of four independent experiments with a total number of cells $n>200$).

stronger in clone S4 compared with clone L1 and stable up to 4 days. Phosphorylation of p38 was not affected (data not shown).

FGFR1 overexpression and leupeptin treatment promotes axonal growth by DRG neurons

So far, the data from PC12 cells indicate that FGF-2 induces neurite outgrowth in response to $FGFR1_L$- and $FGFR1_S$-overexpression and leads to receptor degradation which is blocked by the lysosomal inhibitor leupeptin. We transferred these findings to cultures of adult rat DRG neurons in order to study their relevance for peripheral axon regeneration *in vitro*. Dissociated sensory neurons were transfected with $FGFR1_L$-EGFP, $FGFR1_S$-EGFP or EGFP alone by electroporation using the Amaxa Nucleofector™. The transfection efficiency was 15% for $FGFR1_L$ and $FGFR1_S$, respectively, and 35% for EGFP alone. Neurons identified by EGFP fluorescence were photographed 48 h after transfection. The cultures were then treated with FGF-2 (100 ng/ml+10 μg/ml heparan sulfate) and/or with leupeptin (0.3 mM). After 24 h treatment, the same neurons were documented again and total axonal length and MD of the identified neurons was determined. Only those cells which axons exhibited increased axon length between 48 and 72 h after transfection were taken into consideration. Approximately 10% of neurons in the control and experimental group, respectively, were negatively affected by electroporation (exhibiting a reduced total axon length at day 3 when compared with day 2) and excluded from further analysis.

The total axonal length and the MD of $FGFR_S$ overexpressing neurons increased significantly after 24 h FGF-2 treatment, and this effect was strongly enhanced by co-treatment with leupeptin (Figs. 7 and 8). However, leupeptin alone without FGF-2 had no effect on axonal growth. The effect of FGF-2 on $FGFR1_L$ overexpressing neurons was weaker compared with $FGFR1_S$, but co-treatment with leupeptin intensely stimulated axonal growth by neurons overexpressing either FGFR1 construct. In comparison, none of the treatments affected axonal length of EGFP control transfected cells significantly within the 24 h period. Due to the fact that the transfection efficiency of FGFR1 is merely 15%, overexpression of $FGFR1_L$ and $FGFR1_S$ was confirmed by quantitative single cell PCR of five cells per group (Fig. 9). While in the EGFP control group FGFR1 mRNA could not be detected at the five-cell level, FGFR1 overexpressing neurons revealed C_T-values of 26.6 (±0.7) for $FGFR_S$ and 27.8 (±0.7) for $FGFR_L$. Similar C_T-values of the HRPT1 control gene were observed in all groups and confirmed the same amount of cDNA in each group. The C_T-values together with the agarose separation of the PCR products indicate a clear overexpression of FGFR1 in the $FGFR_S$ and $FGFR_L$ group compared with EGFP-transfected controls which are below the limit of detection at the five-cell level.

The decrease in receptor fluorescence was delayed in FGFR1 overexpressing neurons after treatment with leupeptin and FGF-2 compared with untreated control neurons (Fig. 10). After 4 and 6 day treatment with leupeptin and FGF-2 (starting 48 h after transfection), more neurons expressed receptor fluorescence than in cultures without leupeptin.

DISCUSSION

The present study reveals the specific effect of tyrosine kinase receptor overexpression on promotion of axon regeneration by adult neurons *in vitro*. Exemplified by FGFR1, we show that inhibition of lysosomal degradation results in enhanced receptor stability which leads to a strong induction of adult axon regeneration *in vitro*. FGFRs belong to the neurotrophic receptor family (Baird, 1994) promoting axonal growth during development and regeneration in the adult organism. Their function in neuronal survival and differentiation is highly conserved during evolution. Not only in vertebrates, but in *Drosophila melanogaster* and *Caenorhabditis elegans* as well, FGFs and FGFR homologues are responsible for axon outgrowth and maintenance (Huang and Stern, 2005). The results presented here demonstrate the positive effects of FGFR1 overexpression on neuronal cells leading to enhanced

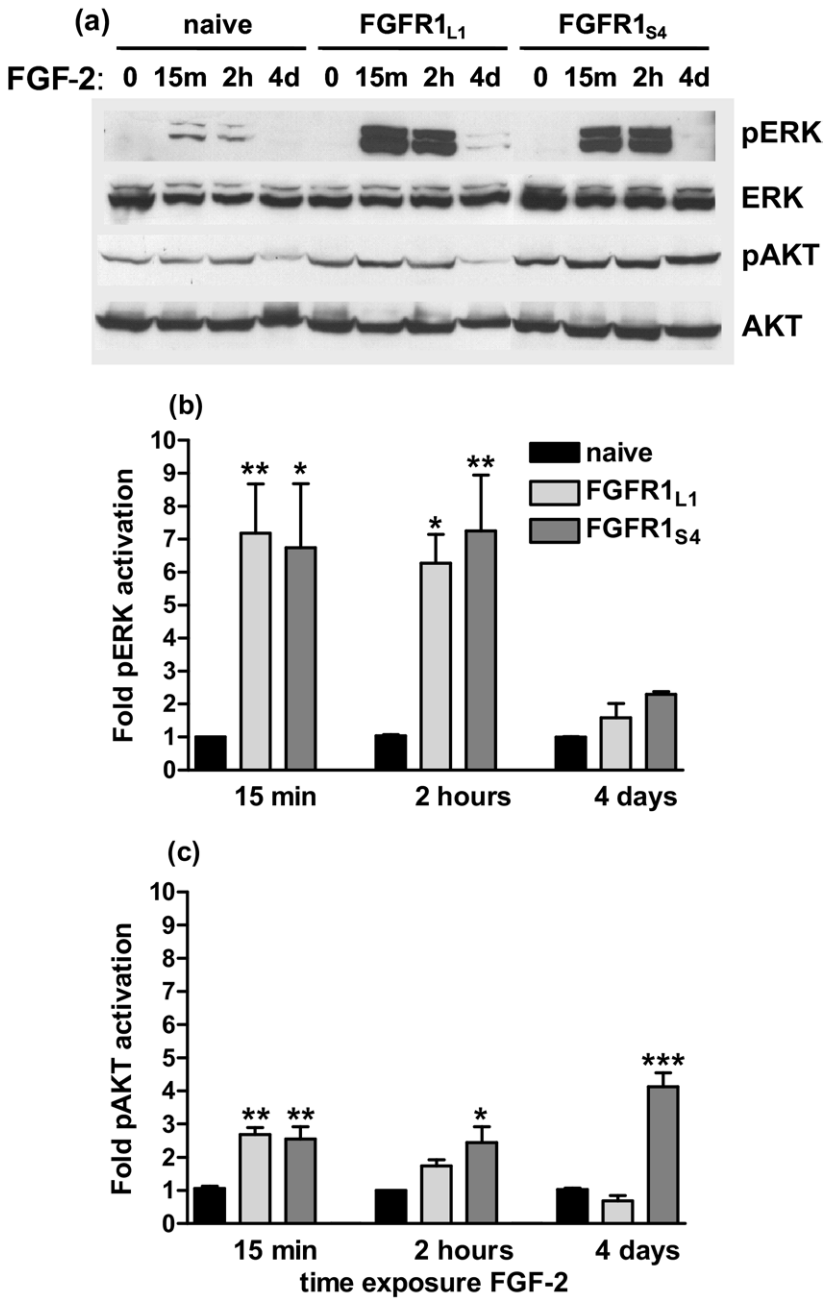

Fig. 6. Intracellular signaling leading to increased neurite outgrowth by FGFR1 overexpressing PC12 cells. (a) Naive and $FGFR1_L$ (L1) or $FGFR_S$ (S4) overexpressing PC12 cell clones were exposed to FGF-2 for 15 min, 2 h or 4 days and lysates were analyzed by Western blotting for phosphorylation of ERK and AKT. Similar results were obtained in three independent experiments. Band intensities of ERK (b) and AKT (c) phosphorylation were densitometrically quantified using Quantity One Software (Biorad) and the ratios of pERK relative to ERK and pAKT relative to AKT were plotted relative to naive cells at given time points. Graphs include data from three independent experiments (asterisks indicate significant differences compared with naive cells, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, one-way ANOVA with Tukey's post test, mean±S.E.M.).

neurite outgrowth by PC12 cells and improved axon regeneration by adult DRG rat neurons *in vitro*.

$FGFR1_L$ and $FGFR1_S$ degradation, monitored by fluorescence measurement, was inhibited by the lysosomal inhibitor leupeptin and by the proteasomal inhibitor lactacystin. Ligand-induced FGFR activation is followed by rapid endocytosis and lysosomal degradation which leads to the termination of the signal (Wong et al., 2002; Bache et al., 2004). A connection between proteasomal and lysosomal degradation has already been identified (Raiborg et al., 2003), however, the exact mechanisms by which the proteasome affects lysosomal receptor degradation is not clear. Proteasome inhibitors prevent lysosomal targeting of EGF (epidermal growth factor) or TrkA receptors by excluding the receptors from entering the intraluminal vesicles of multivesicular endosomes (van Kerkhof et al., 2001;

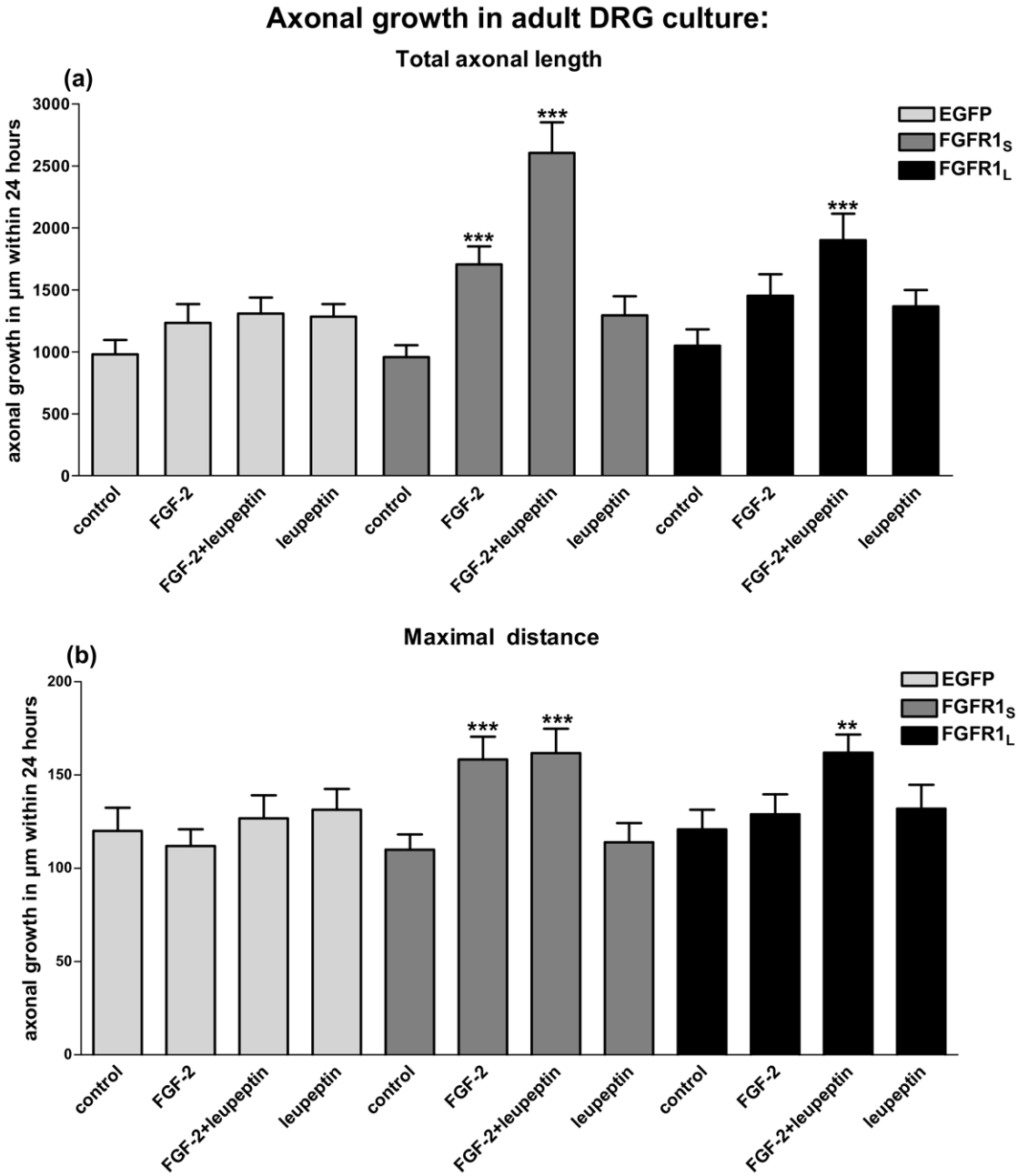

Fig. 7. Transiently transfected adult DRG neurons overexpressing EGFP-fused $FGFR1_S$, $FGFR1_L$ or EGFP alone. Total axonal length (a) and MD (b) of DRG neurons was measured in digitized images. Starting 48 h after transfection, the cultures were treated with 100 ng/ml FGF-2+10 μg/ml heparan sulfate or with 0.3 mM leupeptin or with both FGF-2 plus leupeptin for 24 h (** $P<0.01$, *** $P<0.001$, one-way ANOVA with Tukey's post test, mean±S.E.M. of five independent experiments with a total number of neurons $n>50$).

Longva et al., 2002). It is possible that as yet unknown proteins which serve as negative regulators of sorting into multivesicular endosomes need to be degraded by the proteasome. Evidence exists that such poly-ubiquitinated proteins are associated with endosomes (Bishop et al., 2002).

Stable overexpression of $FGFR1_L$ and $FGFR1_S$ by PC12 cells induced a morphology different from naive PC12 cells. Without growth factor treatment naive PC12 cells exhibit round or ovoid cell bodies. NGF induces cell flattening and the first neurites are detectable after 1 day of treatment (Rydel and Greene, 1987). PC12 cell clones overexpressing $FGFR1_L$ or $FGFR1_S$ exhibited such a flattened, bipolar morphology without growth factor treatment. The proportion of flattened cells is reflected by their potential for FGF-2-induced neuritogenesis. The cells with the highest percentage of flattened cells revealed the most prominent neurite outgrowth in response to FGF-2 treatment. In comparison to FGF-2, NGF induced significantly shorter neurites in $FGFR1_L$ and $FGFR1_S$ overexpressing

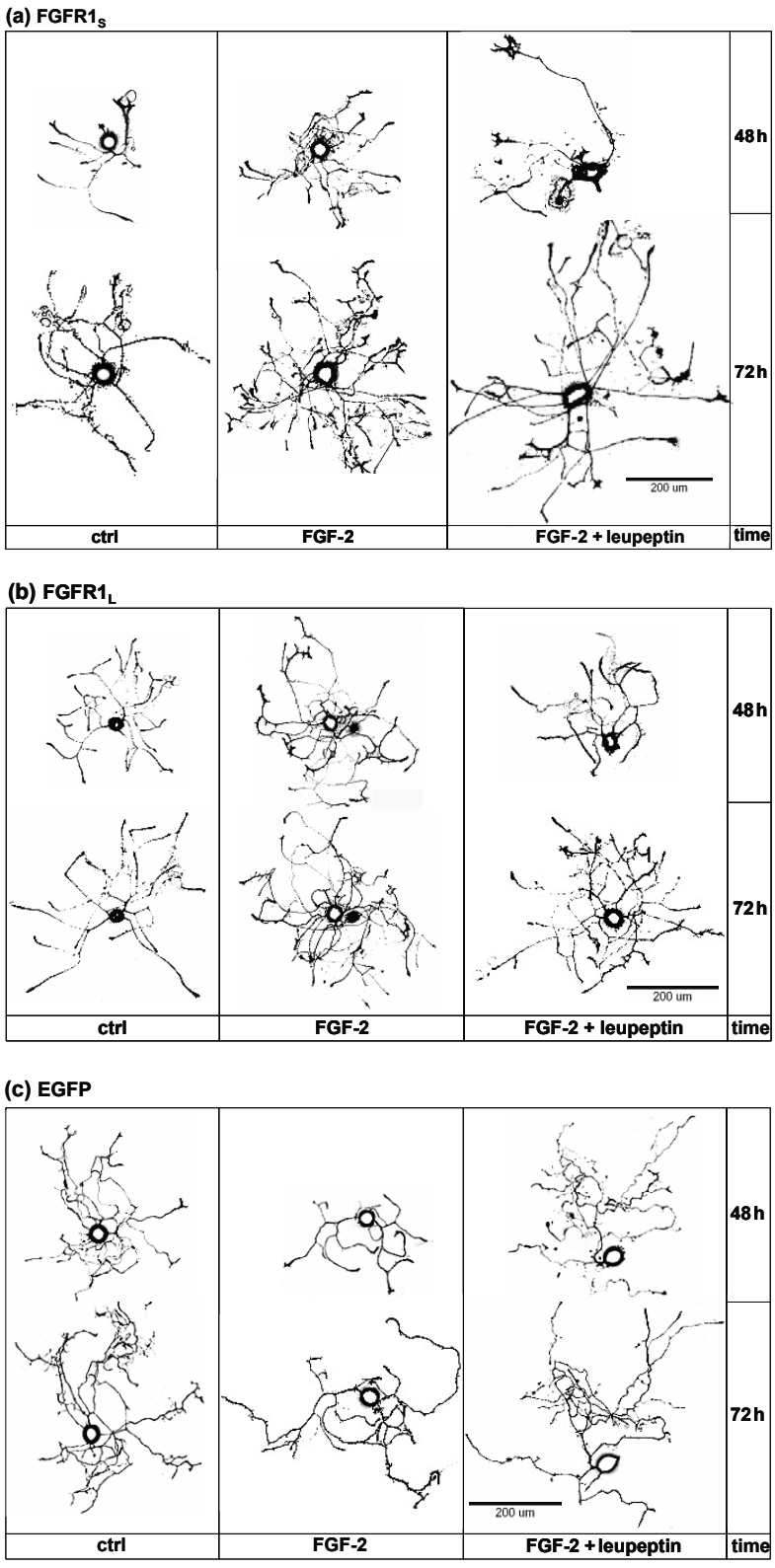

Fig. 8. Representative examples of neuronal morphologies in dissociated adult sensory neuron culture 48 and 72 h after transfection with EGFP-fused $FGFR1_S$ (a), $FGFR1_L$ (b) or EGFP alone (c) and 24 h treatment with FGF-2 (100 ng/ml+10 μg/ml heparan sulfate) or with FGF-2 plus leupeptin (0.3 mM). Inverted fluorescence images are shown after cell body and background correction to document the different axonal lengths and complexities of the axonal tree.

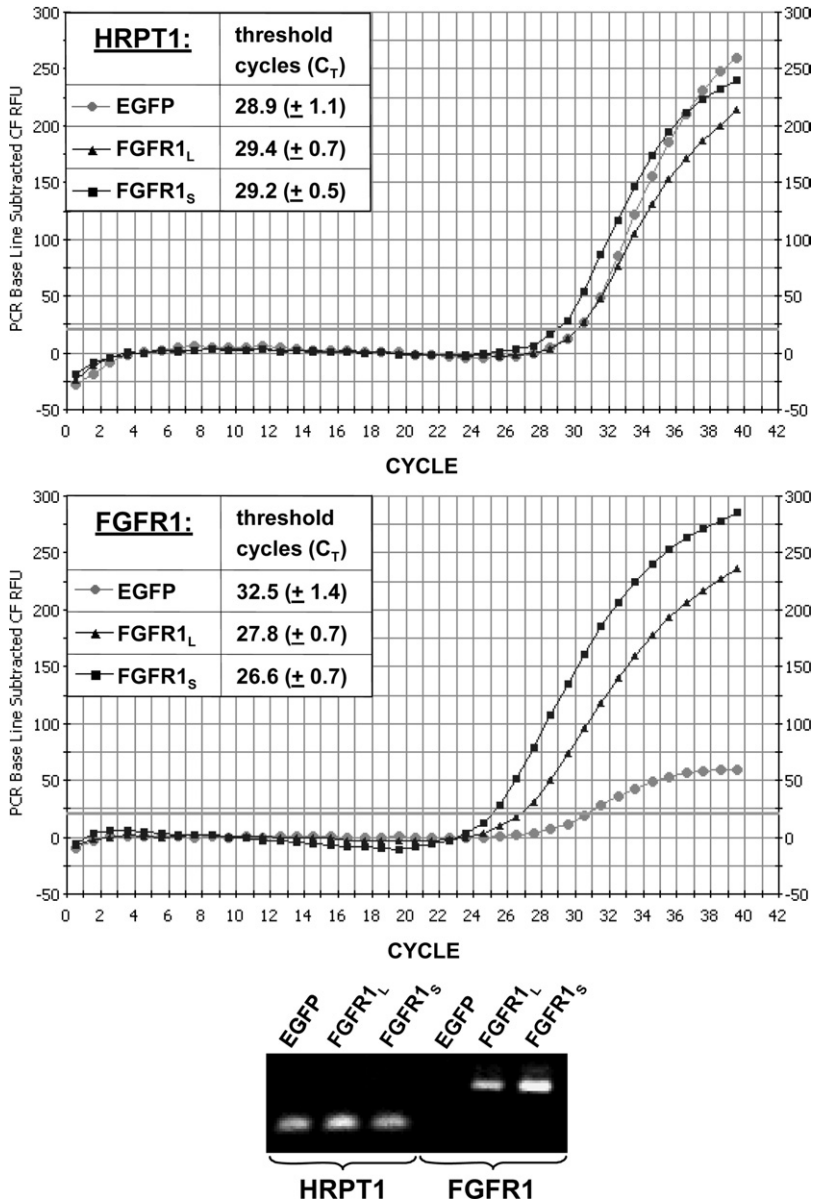

Fig. 9. FGFR1 overexpression by DRG neurons. mRNA expression of FGFR1 by $FGFR1_L$, $FGFR1_S$ and EGFP transfected cells determined by quantitative single cell PCR and agarose gel electrophoresis of five cells per group compared with HRPT1 control gene (n=5).

PC12 cells. Co-treatment with FGF-2 and NGF increased neurite initiation but not neurite length. A crosstalk of FGFR1 and the NGF receptor, TrkA, is indicated by the fact that NGF treatment up-regulates FGFR1 mRNA and makes naive PC12 cells susceptible to FGF-2. Since only the neurite initiation but not the neurite length is affected by co-treatment of FGF-2 and NGF, the way this crosstalk works needs to be explored in detail. PC12 cells overexpressing a dominant negative FGFR reveal reduced NGF-induced neuritogenesis and MAP kinase activation, while selective FGFR inhibitors or oligonucleotides that interfere with FGF-2 receptor binding completely block neurite outgrowth induced by NGF (Chevet et al., 1999). Treatment of PC12 cells with bone morphogenetic protein 2 (BMP2) does not result in neuronal differentiation, but up-regulates FGFR1 on mRNA and protein level rendering PC12 cells responsive to subthreshold concentrations of FGF-2 (Hayashi et al., 2001). These results suggest that activated FGFR1 forms complexes with other growth factor receptors and probably represents a common signaling interface for various extracellular stimuli that regulate neurite outgrowth. FGF-2 activates not only FGFR1 but also FGFR4 and splice variants of FGFR2 (IIIb) and FGFR3 (IIIc). All types of FGFRs are expressed by PC12 cells (Neiiendam et al., 2004), with FGFR1 being expressed at a higher level than FGFR2-4 (data not shown). Since FGFR2-4 levels are not influenced by FGFR1 overexpression (data not shown), the other FGFRs appear not to participate in FGF-2 induced neurite outgrowth after FGFR1 overexpression.

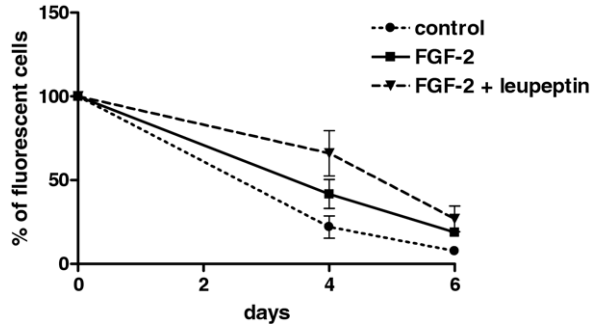

Fig. 10. Proportion of fluorescent neurons overexpressing EGFP-fused $FGFR1_S$ after 4 and 6 day treatment with FGF-2 or leupeptin plus FGF-2, starting 48 h after transfection. Number of fluorescent cells on days 4 and 6 (normalized to the number of fluorescent cells before treatment, mean±S.E.M. of three independent experiments).

ERK and p38 MAP kinase pathway activation is observed in various cell types in response to FGF-2, whereas induction of other kinases like JNK or AKT differs among cell types (Dailey et al., 2005). In PC12 cells, FGFR activation results in sustained ERK activation leading to differentiation, whereas EGFR (epidermal growth factor receptor) activation induces transient ERK activation and proliferation (Traverse et al., 1992). While p38 activation is not involved in FGF-2-induced PC12 cell differentiation, a strong and sustained activation of this MAP kinase mediates FGF-2-induced proliferation in fibroblasts (Maher, 1999). Our results demonstrate that PC12 cell clones overexpressing FGFR1 exhibit intense FGF-2-induced neurite outgrowth accompanied by a strong and sustained activation of ERK without changes in p38 phosphorylation. Furthermore a weaker but even more sustained activation of AKT (up to 4 days) was observed in these cells. FGFR-mediated activation of AKT via PI3-kinase results in protection from apoptosis and cell survival (Dailey et al., 2005), and in the nervous system AKT activation through classical neurotrophins like NGF is necessary and sufficient for survival (Rodgers and Theibert, 2002). NGF induces survival by prolonged activation of PI3-kinase/AKT in PC12 cells (Zhang et al., 2000) and our results demonstrate that AKT is activated by FGF-2 in FGFRl overexpressing PC12 cells as well.

Leupeptin treatment had no effect on neurite outgrowth of $FGFRl_L$ or $FGFR1_S$ overexpressing PC12 cells (data not shown). Accordingly, Saito and Kawashima (1988) reported that leupeptin (Ac-Leu-Leu-Arg-al) did not induce neurite outgrowth by PC12 cells. Other protease inhibitors, like the leupeptin analog Ac-Leu-Leu-Nle-al, induced neuritogenesis which was, however, different from NGF-mediated effects with fewer processes per cell that disappeared after 5 days (Saito and Kawashima, 1989). Application of leupeptin following a neonatal sciatic nerve crush rescues long-term motoneuron survival and improves muscle function (Harding et al., 1996). The same protective effect was observed in motoneuron cultures from E14 embryonic rats against AMPA- (glutamate agonist) induced excitotoxic cell death (Kieran and Greensmith, 2004). Leupeptin increased neurite outgrowth in neonatal mouse DRG tissue culture (Hawkins and Seeds, 1986) and induced axon sprouting in the sciatic nerve (Alvarez et al., 1992, 1995; Moreno et al., 1996). Direct administration of leupeptin into the rat sciatic nerve leads to increased Schwann cell proliferation, demyelination and axonal sprouting, the same alterations which normally follow nerve transection. In this case, protease inhibitors induce axonal sprouting by disrupting a repressive mechanism, modifying the adhesive properties of axons and Schwann cells and altering the functional components of the extracellular matrix. The authors suggest that Schwann cells and a physiological protease which are both inactivated by leupeptin participate in repressing axon sprouting. Those previous studies focused on the extracellular proteolytic effect of leupeptin which is important for interactions of growth cone and extracellular matrix. The present data reveal a new effect of leupeptin on adult axon growth by FGFR1 overexpressing but not by control neurons. As Haugsten et al. (2005) demonstrated, leupeptin inhibits lysosomal FGFR1 degradation and FGFR1 revealed the highest levels of ubiquitination and the fastest degradation of all four types of FGFRs. The prominent effect of leupeptin on FGF-2-induced axon regeneration by adult rat DRG neurons overexpressing FGFR1 supports the hypothesis that blockade of degradation of this tyrosine kinase receptor stimulates adult axon regeneration. Treatment with leupeptin alone had no effect, indicating that its extracellular proteolytic activity is not sufficient for the stimulation of axon growth by adult DRG neurons.

FGFR1, -2 and -4 are expressed differently in the rodent DRG and FGFR3 is up-regulated in response to a nerve lesion (Oellig et al., 1995; Grothe et al., 1997, 2001). We confirmed by quantitative real-time PCR that FGFR1, -2 and -4 are expressed by cultured DRG neurons with FGFR1 being expressed at much higher levels than FGFR2 or FGFR4 (data not shown). The effects of FGF-2 and leupeptin on axonal growth presented in this study seem to be specifically mediated by FGFR1, since there was no effect on EGFP control transfected cells.

Our previous studies revealed that FGF-2 isoforms significantly enhance elongating axon growth by adult sensory neurons in response to a preconditioning sciatic nerve lesion (Klimaschewski et al., 2004). The promotion of axon elongation by FGFR1 overexpression exceeded the elongating effect of a preconditioning lesion. The MD of prelesioned neurons was enhanced by a maximum of about 20% in response to FGF-2 treatment. In comparison, FGF-2 or FGF-2 plus leupeptin treatment increased the MD of $FGFR1_S$ overexpressing neurons up to 50%, the total axonal length was even more enhanced (up to 180%). The elongating properties of FGFR1 overexpressing neurons as indicated by the MD, provide evidence for an enhanced regenerative capacity of adult neurons by tyrosine kinase receptor overexpression. Although much remains to be determined about the cellular mechanisms involved, the present study provides evidence for the importance of enhanced FGFR1 levels for promotion of axon regeneration in the adult nervous system.

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