ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth

Akira Motegi[1,3], Jiro Fujimoto[1], Masaharu Kotani[2], Hitoshi Sakuraba[2] and Tadashi Yamamoto[1,*]

[1]Division of Oncology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan
[2]Department of Clinical Genetics, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Tokyo, 113-8613, Japan
[3]Genome Instability Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892-4442, USA
*Author for correspondence (e-mail: tyamamot@ims.u-tokyo.ac.jp)

Accepted 24 February 2004
Journal of Cell Science 117, 3319-3329 Published by The Company of Biologists 2004
doi:10.1242/jcs.01183

Summary

Anaplastic lymphoma kinase (ALK) is a receptor-type protein tyrosine kinase that is expressed preferentially in neurons of the central and peripheral nervous systems at late embryonic stages. To elucidate the role of ALK in neurons, we developed an agonist monoclonal antibody (mAb) against the extracellular domain of ALK. Here we show that mAb16-39 elicits tyrosine phosphorylation of endogenously expressed ALK in human neuroblastoma (SK-N-SH) cells. Stimulation of these cells with mAb16-39 markedly induces the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), Shc, and c-Cbl and also their interaction with ALK and activation of ERK1/2. Furthermore, we show that continuous incubation with mAb16-39 induces the cell growth and neurite outgrowth of SK-N-SH cells. These responses are completely blocked by MEK inhibitor PD98059 but not by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin, indicating an essential role of the mitogen-activated protein kinase (MAP kinase) signaling cascade in ALK-mediated growth and differentiation of neurons.

Key words: ALK, Agonist monoclonal antibody, Neuroblastoma, MAP kinase, Cell growth, Neurite

Introduction

Many receptor-type protein tyrosine kinases (RTKs) are expressed in the central and peripheral nervous systems and play roles in neural development such as cell proliferation, differentiation, cell survival, and synaptic formation. Small polypeptides known as neurotrophic factors exert their effects by binding to the extracellular region of RTKs (Bibel and Barde, 2000; Chao et al., 1998; Kaplan and Miller, 2000). Upon ligand binding, the receptor induces the intrinsic tyrosine kinase activity and phosphorylates its own specific tyrosine residues. The phosphotyrosine-containing motifs thus created serve as target sites for Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing adaptor molecules such as Shc and IRS-1. The association of Shc with the Grb2/Sos complex links RTK to the ras/MAP kinase signaling cascade and thus transmits signals (Kazlauskas, 1994; Sclessinger, 2000).

Anaplastic lymphoma kinase (ALK) was originally identified as an oncogene activated in anaplastic large cell lymphomas (ALCL) with chromosomal translocation t(2;5) (Shiota et al., 1994, 1995). In one-third of cases of this subset of non-Hodgkin's lymphoma, the cytoplasmic domain of ALK is fused to the amino terminal half of nucleophosmin (NPM) to generate a fusion gene product, $p80^{NPM\text{-}ALK}$ (Morris et al., 1994). NPM is a multifunctional protein that plays a role in protein shuttling between the cytoplasm and nucleus (Borer, at al., 1989) and in cell cycle-dependent centrosome duplication (Okuda et al., 2000). Previous studies have shown that the NPM portion of $p80^{NPM\text{-}ALK}$ mediates oligomerization, leading to constitutive activation of ALK and downstream effector molecules, thereby inducing cellular transformation (Bischof et al., 1997; Fujimoto et al., 1996). Subsequent cDNA cloning of the full-length proto-*alk* have shown that it encodes a novel, putative RTK of the insulin receptor family (Iwahara et al., 1997; Morris et al., 1997).

The most closely related kinase to ALK is leukocyte tyrosine kinase (LTK), which lacks a sequence corresponding to the amino terminal half of the ALK extracellular domain (Ben-Neriah and Bauskin, 1988; Krolewski and Dalla-Favera, 1991). The extracellular domain and protein kinase domain of LTK possess 50% and 78% amino acid identity, respectively, to the corresponding regions of ALK. These two kinases comprise a new subfamily of the insulin receptor family. In situ hybridization analysis has shown that *alk* is expressed almost exclusively in perinatal neural cells. In particular, *alk* transcripts are highly expressed in specific regions of the central nervous system, including the diencephalon, midbrain, and the ventral half of the spinal cord. In the peripheral nervous system, expression of *alk* has been identified in the trigeminal, sympathetic, and enteric ganglia. After birth, expression decreases, but persists in some regions such as the thalamus, olfactory bulb, and mesencephalon (Iwahara et al., 1997).

Despite the potential importance of ALK in the development of the nervous system, little is known about its cognate ligand(s) except recent studies proposing that pleiotrophin (PTN) and midkine (MK) are ligands for ALK (Stoica et al., 2001; Stoica et al., 2002). PTN and MK are secretory heparin-binding growth and differentiation factors that exert a variety of effects

in normal development and tumor growth, including mitogenic, neuritogenic, angiogenic, and morphogenetic activity (Muramatsu, 2002). In contrast to the restricted expression of *alk* in the nervous system, *ptn* and *mk* transcripts are distributed widely in both neural and non-neural embryonic tissues (Mistiadis et al., 1995). Both PTN and MK also bind receptor-type protein tyrosine phosphatase-ζ/β (RPTPζ/β) (Maeda et al., 1996; Maeda et al., 1999) and syndecans (Kojima et al., 1996; Raulo et al., 1994), ubiquitously expressed proteoglycans, suggesting that these molecules may participate in some PTN/MK activities. Given the possible multiplicity of ligand-receptor interactions, it will be useful to develop receptor-specific agonists to understand the exact output of each receptor.

Monoclonal antibodies (mAbs) against cell surface receptors have been used widely to study the mechanisms of receptor-ligand interactions and receptor activation (Ashman et al., 1994; Fernandez-Pol, 1985; Forsayeth et al., 1987; LeSauteur et al., 1996; Prat et al., 1998; Schreiber et al., 1981; Soos et al., 1989; Spaargaren et al., 1991; Stancovski et al., 1991; Sunada et al., 1986; Xiong et al., 1992; Yarden, 1990). When a well-characterized ligand is not available, agonist mAbs have been shown to be effective tools in the analysis of receptor function (Stancovski et al., 1991; Yarden, 1990). Here we describe the development and characterization of an agonist monoclonal anti-ALK antibody termed 16-39 directed against the extracellular domain of ALK. With this antibody, we showed that ALK transmits both mitogenic and differentiation signals, and that the ERK/MAPK pathway plays an important role in these effects in SK-N-SH human neuroblastoma cells.

Materials and Methods

Reagents, cell lines and antibodies

Murine 2.5S NGF was purchased from Takara Biomedicals (Tokyo, Japan). ITS supplement (insulin, transferrin, and selenium) and geneticin (G418) were obtained from Gibco Life Technologies (Rockville, MD). PD98059 and wortmannin were from Calbiochem (San Diego, CA) and Sigma (St Louis, MO), respectively. To obtain 2-C4 cells, NIH 3T3 cells were transfected with pME-*alk* (Iwahara et al., 1997) and pCMV-neo plasmids, containing cDNA of mouse ALK and the neomycin resistant gene, respectively, by using Superfect transfection reagent (Qiagen), and G418-resistant cells were isolated. The human neuroblastoma cell line SK-N-SH was obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in minimum essential medium-α (MEM-α) supplemented with 10% fetal bovine serum (FBS). NIH 3T3 and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Polyclonal anti-mouse ALK antibodies were prepared by immunizing rabbits with glutathione *S*-transferase (GST) fusion protein containing the amino acid residues 1503-1621 of mouse ALK (GST-ALK). To purify anti-ALK antibodies, antisera were successively passed on GST- and GST-ALK-coupled columns (HiTrap NHS-activated, Amersham). Anti-phosphotyrosine (PY) antibody conjugated with HRP (RC20-HRP) was purchased from Transduction Laboratory (Lexington, KY). Anti-phospho-ERK1/2 antibody (Cat. No. 9101) was from New England Biolabs, Inc. (Beverly, MA). Antibodies recognizing ERK1/2, c-Cbl, PLCγ and myc-epitope tag (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Shc (Cat. No. 06-203) and anti-IRS-1 antibodies were from UBI (Lake Placid, NY). Polyclonal antibodies against the p85 subunit of PI 3-kinase were kindly provided by Dr Fukui (University of Tokyo). Antibody against rat IgG $F(ab')_2$ conjugated with HRP was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The normal human and rat IgG were from Zymed Inc. (San Francisco, CA).

Plasmid constructions

The construction of plasmids expressing the entire extracellular domain of mouse ALK with cleavable myc-His tag at carboxyl terminus (pAPmH) was carried out as follows: The *Hin*dIII site was introduced after the last codon of extracellular domain of mouse *alk* by PCR with the mutagenizing primer: 5′-ATCAATGAGAGAA-GCTTGTGGGGCTC-3′. The resulting *Not*I-*Hin*dIII fragment (*Not*I from the vector) of *alk* and the *Hin*dIII-*Bst*EII fragment encoding the PreScission protease (Amersham) cleavage site (consisting of two oligonucleotides; 5′-AGCTTCTGGAAGTTCTGTTCCAGGGG-CCCG-3′ and 5′-GTGACCG-GGCCCCTGGAACAGAACTTCC-AGA-3′) were ligated into the *Not*I and *Bst*EII sites of a mammalian expression vector, pcDNA3.1/myc-His version C (Invitrogen). For the construction of an expression vector encoding ALK-Fc, the cDNA of extracellular domain of mouse *alk* was ligated in-frame to the fragment that encodes Fc portion of human IgG. The resulted fragment was subcloned into the mammalian expression vector pME18S.

Expression and purification of immunogen

The pAPmH plasmid was transiently transfected into HEK 293T cells (1.5×10^6 cells per 100 mm dish) by the standard calcium phosphate method. The cells were cultured in serum-reduced (1%) medium for 4-5 days. The recombinant protein expressed in conditioned medium was collected on the chelating Sepharose resin (HiTrap, Amersham). The beads were washed with PBS and then equilibrated with cleavage buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). The bound protein was eluted by cleaving with PreScission protease (used at 2 U/μg of protein) for 2 hours at 4°C. The protease itself is a GST-fusion protein and was also removed from the eluate by passing through glutathione-Sepharose beads (Amersham). The recombinant ALK protein thus obtained was dialyzed against PBS and used for immunization. The final yield was 0.5 μg/ml of conditioned medium. The ALK-Fc protein was expressed in the same way as above and collected on protein A column (HiTrap, Amersham). After extensive washing, the protein was eluted with 100 mM Na citrate (pH 3.0) and dialyzed against PBS.

Immunization, cell fusion and screening by enzyme-linked immunosorbent assay (ELISA)

A three-week-old female rat was injected in the footpads firstly with 50 ng antigen plus adjuvant, and then twice with 10 ng antigen without adjuvant at 6-day intervals. Three days after the last injection, popliteal lymph nodes were removed and the cells were fused with PAI myeloma cells (Kotani et al., 1993). ELISA screening was performed to identify positive hybridoma supernatants. Either 20 ng of ALK-Fc or normal human IgG was absorbed on 96-well microtiterplates (MTP). 100 μl hybridoma supernatants were incubated for 45 minutes at room temperature. After washing four times, anti-rat IgG $F(ab')_2$ antibody conjugated with HRP was added, incubated for another 45 minutes and washed again. The peroxidase substrate, *O*-phenylenediamine, was added and OD_{405} of each well was measured with an MTP reader. Clones that reacted to ALK-Fc but not to human IgG were judged as positive. After secondary selection according to the result of stimulation assay, the clones of interest were recloned by limiting dilution.

Immunoblotting and immunoprecipitation analyses of mAb16-39

For immunoblotting analysis, APmH protein prepared as described above (without protease digestion) was separated by 7.5% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore). Membranes were incubated with anti-myc or purified mAb16-39 antibodies (2 μg/ml each), and then,

with anti-rat or anti-rabbit IgG F(ab′)$_2$ secondary antibody conjugated with HRP, respectively. Specific bands were visualized with enhanced chemiluminescence (Renaissance, Perkin Elmer, Boston, MA). For immunoprecipitation analysis, APmH-containing media were mixed with either purified mAb16-39, anti-myc antibody (9E10), or control IgG, (2 μg each) and the immunoprecipitates were collected on protein G-Sepharose beads (Amersham). As a control, APmH protein was mixed with chelating Sepharose (Amersham) from equal amounts of APmH-containing medium. The immunoprecipitated APmH protein was detected with anti-myc antibody (9E10).

Large scale preparation of mAb16-39

Hybridoma clone 16-39 was cultured in a gas permeable bag (i-Mab kit, Diagnostic Chemicals, Charlottetown, PE, Canada) in 500 ml PRMI medium for up to 1 week. Supernatant was collected and passed over a protein G column (HiTrap, Amersham). After extensive washing, bound antibody was eluted with 0.1 M glycine (pH 2.7) and immediately neutralized with 1 M Tris-HCl (pH 9.5). Eluate was dialyzed against PBS and then the purity and protein concentration were estimated by SDS-PAGE and BCA protein assay kit (Pierce, Rockford, IL).

Stimulation assays

SK-N-SH or 2-C4 cells (1×10^6) were seeded into 60-mm dishes. When cells were subconfluent, dishes were washed once with serum-free media and serum-free media supplemented with ITS (insulin, transferrin, and selenium) was added for serum starvation. After 24 hours, cells were treated with mAb16-39 or control rat IgG at the concentrations indicated in each figure. Cells were then washed three times with PBS to remove mAb and lysed in TNE lysis buffer. Clarified cell lysates were incubated with indicated antibodies for 2 hours at 4°C. To minimize the involvement of mAb for the immunoprecipitation, the immune complexes were collected on protein A-Sepharose beads that bind rat IgG at a negligible level. The proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Some membranes were then stripped by incubating in the buffer (100 mM Tris-HCl pH 7.4, 2% SDS, and 10 mM 2ME) for 30 minutes at 50°C. Then membranes were reprobed with the next antibodies.

Thymidine incorporation assay

2-C4 and NIH 3T3 (1×10^5) or SK-N-SH cells (2×10^5) were seeded in 6-well plates. After incubating in 0.1% serum-containing media for 24 hours, cells were treated with NGF, mAb16-39, or control IgG at the concentrations indicated in the figures and cultured for 48 hours. The MEK inhibitor PD98059 (50 μM), PI 3-kinase inhibitor wortmannin (100 nM), or vehicle (DMSO) was added where indicated 1 hour prior to the addition of NGF or antibodies. Wortmannin was then added on the next day. For the last 4 hours, 1 μCi of [^{3}H]thymidine (87 Ci/mmol, Amersham) was added. Cells were fixed with 10% TCA and lysed with 0.5 N NaOH for 1 hour at 37°C. The amount of incorporated thymidine was determined with a liquid scintillation counter.

Neurite outgrowth of SK-N-SH cells

SK-N-SH cells (5×10^5) were plated in 35-mm dishes and cultured overnight in MEM-α with 10% FBS and ITS. After washing in media without serum three times, cells were cultured in ITS-supplemented media with NGF (2 nM), mAb16-39 (4 nM), or control IgG (4 nM). Pharmacological inhibition of MEK and PI 3-kinase was performed as above. Cells were photographed at day 2.5 under a phase-contrast microscope (Nikon). Cells that extend processes longer than twice the diameter of the cell body were counted as differentiated. Scores at day 0.5, 1.5, and 2.5 were obtained from three low-power fields from at least three independent experiments and expressed as the mean±s.e.m. (%).

Results

Production and screening of anti-ALK monoclonal antibodies

To examine the biological and biochemical functions of endogenous ALK, we developed monoclonal anti-ALK antibodies that recognize the extracellular domain of ALK. Recombinant protein comprising the entire extracellular region of mouse ALK tagged with myc-His epitopes at the carboxyl terminus (APmH, Fig. 1A) was prepared and used as immunogen. The supernatants of hybridoma clones were examined for reactivity against ALK-Fc by the ELISA method. Twelve out of 210 clones were identified as positive (data not shown). As anti-rat IgG antibody was used as a secondary antibody in the ELISA screening, all mAbs obtained

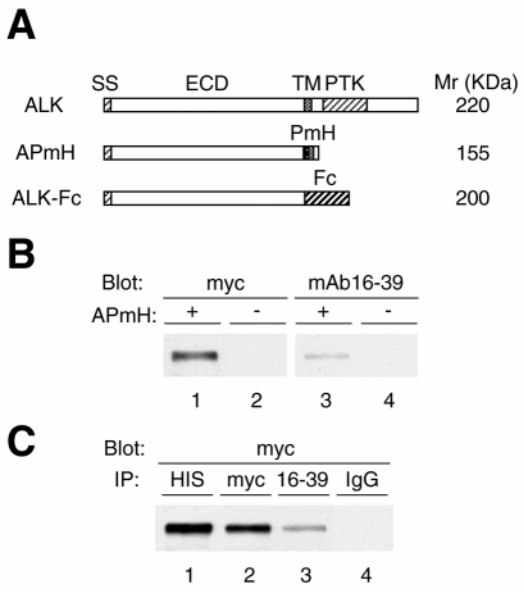


Fig. 1. Characterization of anti-ALK monoclonal antibody. (A) Preparation of immunogen (APmH). The entire extracellular domain (ECD) of mouse ALK (ALK) followed by a PreScission protease site and a myc-His tag (PmH) was expressed and collected as described in Materials and Methods. The ECD of ALK fused with the Fc portion of human immunoglobulin (ALK-Fc) was used as an antigen for ELISA screening. Abbreviations: SS, signal sequence; TM, transmembrane domain; PTK, protein tyrosine kinase domain. (B) HEK 293T cells were transfected with APmH-expressing vector (+) and the recombinant protein was collected on the chelating Sepharose, separated by SDS-PAGE, and transferred to PVDF membranes for immunoblotting. Supernatant from cells transfected with empty vector was treated in the same way as a negative control (–). The membranes were blotted with either anti-myc (myc) or anti-ALK monoclonal (mAb16-39) antibodies (2 μg/ml). (C) APmH protein was immunoprecipitated with anti-myc antibody (myc), mAb16-39 (16-39), or normal rat IgG (IgG) (2 μg each). As a control, the APmH protein was collected by the chelating Sepharose (HIS), which allowing the purification of the recombinant protein through binding to the His tag epitope. Immunoprecipitated proteins were probed with anti-myc antibody 9E10 (myc).

were IgGs. To examine whether these mAbs stimulate autophosphorylation of ALK in intact cells, we generated NIH 3T3 cells stably expressing mouse ALK (designated as 2-C4 cells). Quiescent 2-C4 or parental NIH 3T3 cells were treated with mAb-containing supernatants for 5 minutes at 37°C. ALK protein was immunoprecipitated with polyclonal anti-ALK antibodies that recognize the carboxyl terminus of ALK and immunoblotted with anti-phosphotyrosine antibody. Surprisingly, four out of 12 clones enhanced the tyrosine phosphorylation of ALK at various levels (data not shown). Among them, a clone of the highest activity (no. 16) was recloned and used in the further studies.

Characterization of monoclonal anti-ALK antibody 16-39

To verify the specificity of the selected monoclonal antibody, termed mAb16-39, western blotting and immunoprecipitation analyses with mAb16-39 were performed. The APmH protein was collected on the chelating Sepharose, subjected to SDS-PAGE, and then immunoblotted with anti-myc or mAb16-39 antibodies. Both antibodies detected a band of 155 kDa. This band was not identified in the samples prepared from cells transfected with expression vector alone (Fig. 1B). To examine the binding properties of mAb16-39 to the extracellular domain of ALK further, immunoprecipitation analysis with APmH protein was performed. As shown in Fig. 1C, mAb16-39 immunoprecipitated APmH protein, although the efficiency seemed to be low compared with anti-myc antibody (9E10).

mAb16-39 induces tyrosine phosphorylation of ALK and cellular proteins

Immunoprecipitation and immunoblotting experiments with 2-C4 cells showed two species of ALK (~220 kDa and 140 kDa; Fig. 2, lower panel, lane 2), as reported previously (Iwahara et al., 1997; Morris et al., 1997). In addition, an 85-kDa species was identified (another band a little larger than 85 kDa was non-specific, as it was also observed in the lane of NIH 3T3 cells; Fig. 2, lower panel, lane 1 and unpublished data). Stimulation assay as described above with purified antibody (20 nM) showed that mAb16-39 significantly enhanced the tyrosine phosphorylation of the 220-, 140-, and 85-kDa species of ALK (Fig. 2, upper panel, lane 3). An increase in tyrosine phosphorylation of co-immunoprecipitated 66-, 52-, and 46-kDa proteins was also observed. The presence of equal amounts of ALK in each lane was confirmed by reprobing the same blot with polyclonal anti-ALK antibodies (Fig. 2, lower panel, lanes 2, 3). No detectable tyrosine phosphorylation of anti-ALK precipitates was identified in 2-C4 cells treated with normal rat IgG or in parental NIH 3T3 cells treated with mAb16-39. These results clearly indicate that tyrosine phosphorylation of ALK is a consequence of the specific binding of mAb16-39 to ALK.

ALK is expressed in the mouse embryonic nervous system (Iwahara et al., 1997; Morris et al., 1997) and also in several human neuroblastoma-derived cell lines, including SK-N-SH (Lamant et al., 2000). We examined whether mAb16-39 stimulates tyrosine phosphorylation of endogenously expressed ALK in SK-N-SH cells. As shown in Fig. 2, mAb16-39 cross-reacted with human ALK and induced tyrosine

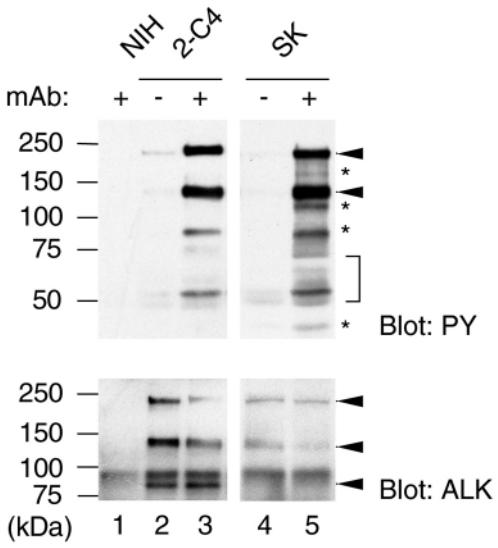


Fig. 2. Monoclonal anti-ALK antibody 16-39 induces tyrosine phosphorylation of ALK. Untransfected NIH 3T3 cells (NIH), derivative cells expressing mouse ALK (2-C4), or human neuroblastoma cells SK-N-SH (SK) were treated with 20 nM normal rat IgG (–) or mAb16-39 (+) for 5 minutes at 37°C. Lysates were immunoprecipitated with polyclonal anti-ALK antibodies that recognize the carboxyl terminus of ALK. The phosphotyrosine level was examined by western blotting (Blot) with anti-PY antibody RC20-HRP (upper panels). The presence of equal amounts of ALK was confirmed by reprobing with polyclonal anti-ALK antibodies (lower panels). Arrowheads indicate the positions of endogenous and transfected ALK (220, 140, and 85 kDa in 2-C4 cells and 220 and 140 kDa in SK-N-SH cells). The bracket shows the position of phosphoproteins of 66, 52, and 46 kDa in both 2-C4 and SK-N-SH cells. Asterisks indicate phosphoproteins of 160, 120, 85, and 35 kDa in SK-N-SH cells.

phosphorylation of ALK and a set of proteins (66, 52, and 46 kDa) similar to those observed in 2-C4 cells. In addition, tyrosine phosphorylation of 160-, 120-, 85-, and 35-kDa proteins was also detected in anti-ALK immunoprecipitates from SK-N-SH cells (Fig. 2, upper panel, lane 5). Because the 85-kDa species of ALK was not detected with anti-ALK antibodies in SK-N-SH cells (Fig. 2, lower panel), the tyrosine phosphorylated 85-kDa band found in SK-N-SH cells may be different from that observed in 2-C4 cells.

The kinetics of mAb16-39-induced tyrosine phosphorylation of ALK were analyzed to characterize mAb16-39 further. 2-C4 cells were incubated with increasing concentrations of mAb16-39, ranging from 0.12-120 nM, for 5 minutes at 37°C. As shown in Fig. 3A, tyrosine phosphorylation of ALK was identified at concentrations as low as 0.4 nM mAb16-39 and was elevated as the concentration increased. When 2-C4 cells were incubated with 4 nM mAb16-39 for varying periods of time, maximal phosphorylation was identified within 5 minutes, and weak phosphorylation could still be detected after 24 hours (Fig. 3B). The amount of the 220-kDa ALK species was slightly decreased after mAb16-39-mediated activation and remained almost constant, whereas that of the 140-kDa species was markedly decreased only after prolonged exposure to the antibody (24 hour, Fig. 3B). These observations suggest that

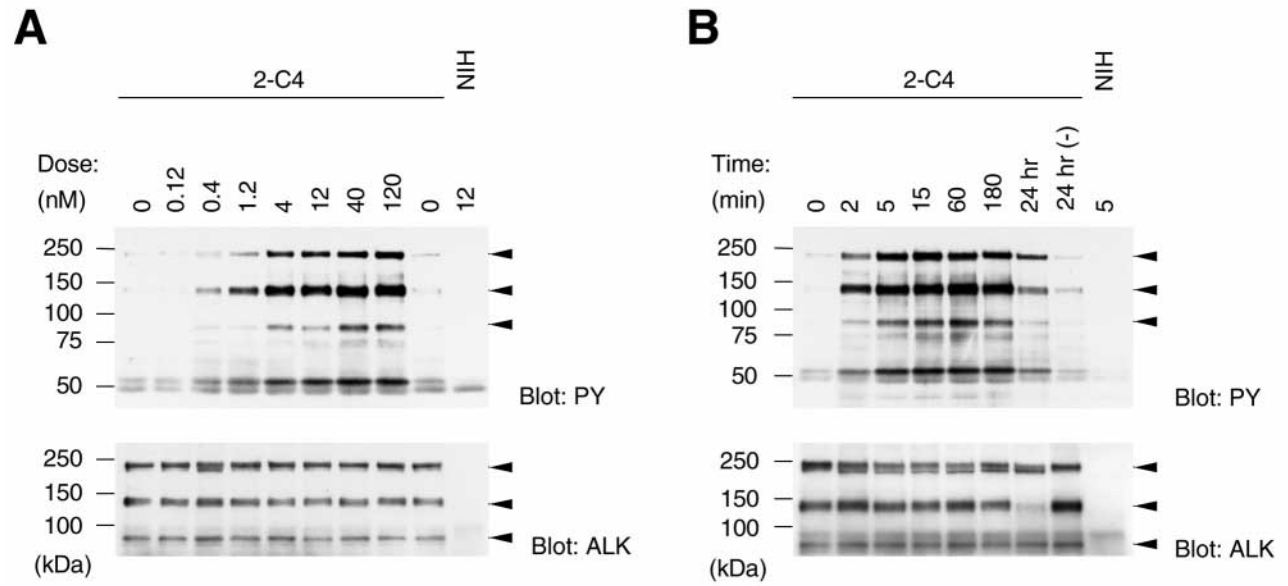


Fig. 3. Dose-response (A) and time course (B) of ALK tyrosine phosphorylation in 2-C4 cells. For dose-response analysis, 2-C4 cells were treated with increasing concentrations of mAb16-39 (0, 0.12, 0.4, 1.2, 4, 12, 40, and 120 nM) for 5 minutes at 37°C. As a control, parental NIH 3T3 cells were treated with 12 nM mAb for 5 minutes. For time-course analysis, cells were incubated with 4 nM mAb16-39 for the periods indicated (0, 2, 5, 15, 60, and 180 minutes and 24 hours). As controls, 2-C4 or parental NIH 3T3 cells were treated with 4 nM control IgG for 24 hours or 4 nM mAb for 5 minutes, respectively. ALK immunoprecipitates were probed with anti-PY antibody RC20-HRP (PY) and then reprobed with anti-ALK antibodies (ALK). The positions of 220-, 140-, and 85-kDa ALK bands are indicated by arrowheads.

the two species of ALK may be differentially processed after ligand binding. Similar results were obtained with SK-N-SH cells (data not shown).

mAb16-39 stimulates tyrosine phosphorylation of signaling molecules and their association with ALK

Previous studies have shown that $p80^{NPM\text{-}ALK}$, an oncogenic form of ALK, is associated with signaling molecules including Shc, IRS-1 (Fujimoto et al., 1996), PLCγ (Bai et al., 1998), and the p85 subunit of PI 3-kinase (Bai et al., 2000; Slupianek et al., 2001). Upon mAb16-39 stimulation, at least seven tyrosine phosphoprotein bands were identified in anti-ALK immunoprecipitates from SK-N-SH cell lysates (Fig. 2, Fig. 4A), suggesting that a similar set of adaptor and/or effector molecules could be utilized in mAb16-39-mediated ALK activation as in $p80^{NPM\text{-}ALK}$ activation. To identify which signaling proteins are involved, co-immunoprecipitation experiments were performed. As shown in Fig. 4A, treatment of SK-N-SH cells with 20 nM mAb16-39 for 5 minutes increased the tyrosine phosphorylation of Shc (66-, 52-, and 46-kDa species) and its association with ALK. To exclude the possibility that anti-ALK mAb is involved in the immunoprecipitation of ALK, control experiments without anti-Shc or anti-ALK polyclonal antibodies were performed. As shown in Fig. 4A (lanes 3 and 6), anti-ALK mAb immunoprecipitated only residual amounts of ALK. Similar experiments with IRS-1 revealed that IRS-1 is also involved in mAb-mediated ALK signaling (Fig. 4B). Because c-Cbl, an adaptor protein that plays a key role in the ubiquitin-mediated downregulation of receptors, is frequently recruited to RTKs upon ligand stimulation (Levkowitz et al., 1999; Soltoff and Cantley, 1996; Yokouchi et al., 1999), we examined whether the 120-kDa band could be c-Cbl. Anti-c-Cbl immunoprecipitates from mAb16-39-stimulated lysates were immunoblotted with anti-PY antibody. c-Cbl was phosphorylated on tyrosine and associated with ALK upon mAb16-39 stimulation (Fig. 4D). Finally, immunoblotting of anti-p85 and anti-PLCγ immunoprecipitates with anti-PY antibody revealed that these proteins were not tyrosine phosphorylated but were recruited to ALK in a mAb16-39 stimulation-dependent manner (Fig. 4C,E). In addition, phosphoproteins of 200, 130, 95, and 70 kDa were identified in anti-p85 immunoprecipitates from mAb16-39-stimulated lysates (Fig. 4C).

mAb16-39 promotes DNA synthesis

As mAb16-39 has such a strong effect on ALK-mediated signals, we sought to determine the cellular effects of the antibody on ALK-expressing cells. To examine whether the antibody stimulates DNA synthesis, a [^{3}H]thymidine incorporation assay was performed. 2-C4 or NIH 3T3 cells were made quiescent by culturing under reduced serum conditions (0.1%) for 24 hours and they were then treated with mAb16-39 or control IgG. As shown in Fig. 5A, treatment of 2-C4 cells with mAb16-39 in a concentration range of 0.8-20 nM induced DNA synthesis comparable to that observed in the presence of 5% serum. Cells treated with control IgG did not show any detectable response, nor did parental NIH 3T3 cells treated with mAb16-39. In SK-N-SH cells, NGF induced an approximate 1.5-fold increase in thymidine uptake, in agreement with previously reported findings (Janet et al., 1995). Remarkably, mAb16-39 also exerted a significant mitogenic effect (2.5 times that of the control) in these cells (Fig. 5B).

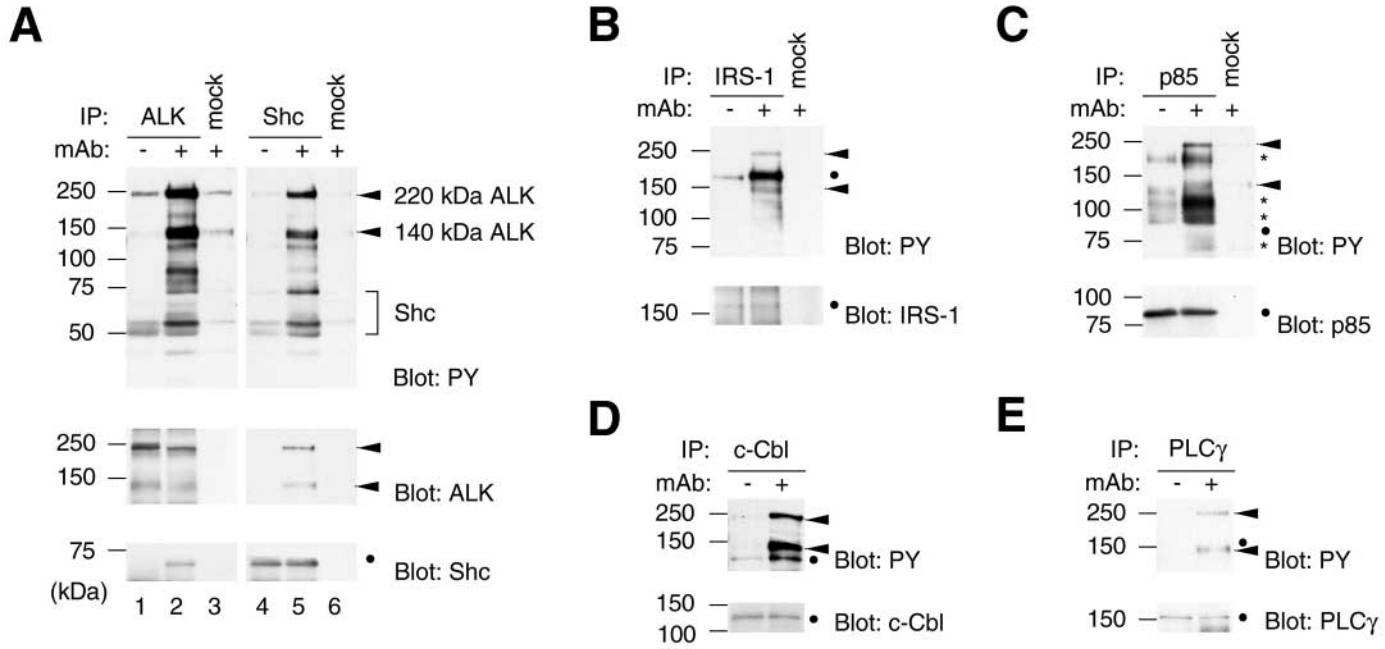


Fig. 4. Analysis of downstream signaling. (A) Serum-starved SK-N-SH cells were stimulated with 4 nM anti-ALK mAb16-39 for 5 minutes. Lysates were immunoprecipitated (IP) with anti-ALK (ALK) or anti-Shc (Shc) antibodies or were immunoprecipitated without antibodies (mock) and immunoblotted with anti-PY antibody (upper panels). The same blots were reprobed with anti-ALK (middle panels) or anti-Shc (lower panels) antibodies. The positions of ALK (220 and 140 kDa) and three isoforms of Shc (66, 52, and 44 kDa) are indicated by the arrowheads and bracket, respectively. The dot shows the position of Shc (66 kDa). (B,C,D,E) Lysates prepared as above were immunoprecipitated with antibodies as indicated and immunoblotted with anti-PY antibody (upper panels) and then reprobed with the indicated antibodies (lower panels). The positions of the immunoprecipitated proteins are indicated by dots. Asterisks in C show unidentified co-immunoprecipitated proteins with anti-p85 antibody.

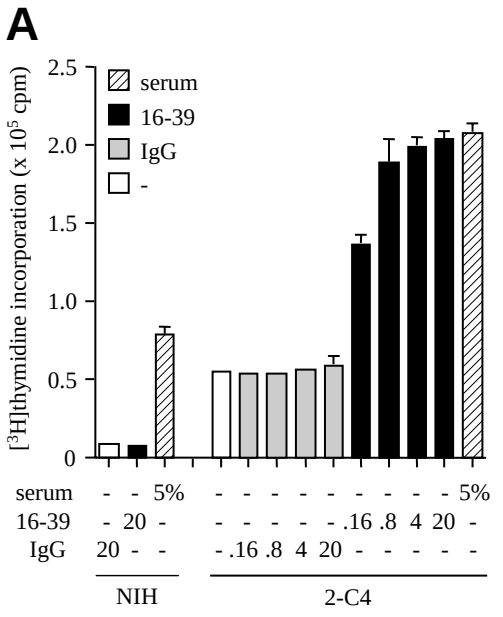
MEK inhibitor blocks the mitogenic effect of mAb16-39

ERK1/2, key molecules of the Ras/MAP kinase pathway, are activated by a variety of RTKs and play important roles in mediating mitogenic and differentiation signals (Boulton et al., 1991; Grewal et al., 1999). To examine whether ERK1/2 are activated in response to mAb16-39, quiescent 2-C4 cells were treated with 4 nM control IgG or mAb16-39 for 5 minutes at 37°C. Immunoblotting of total lysates with anti-phospho-ERK1/2, which reflects the activation state of these kinases, showed that the phosphorylation level of ERK1/2 was clearly enhanced (13-fold) in mAb-treated cells (Fig. 6A,B). Next, to determine whether MAP kinases are involved in mAb16-39-induced DNA synthesis, 2-C4 cells were incubated with mAb16-39 in the presence of the MEK inhibitor PD98059, and [^{3}H]thymidine incorporation was assessed. In the absence of mAb-stimulation, PD98059 inhibited a basal level presumably contributed by the reduced serum (0.1%). Significantly, mAb-stimulated thymidine incorporation was completely suppressed by PD98059 (50 μM), whereas the PI 3-kinase inhibitor wortmannin (100 nM) had no apparent effect on the mitogenic property of mAb16-39 (Fig. 6C).

mAb16-39 induces MAPK-dependent neurite outgrowth

NGF induces the extension of neurites without apparent inhibition of cell growth in SK-N-SH cells (Burchill et al., 1995). To investigate whether mAb16-39 induces morphological changes in SK-N-SH cells, serum-starved cells were cultured in the presence of NGF, mAb16-39, or control IgG and the morphological appearance was observed for a period of 1 week. In response to media with 10% serum, most SK-N-SH cells showed a neuroblast phenotype with round or slightly flattened cell bodies and short- to moderate-length fine processes (data not shown). Upon switching to serum-free media plus control IgG, they showed a tendency to form small clumps, and some of them (less than 20%) extended short neurites (Fig. 7A). In contrast, NGF- or mAb-treated cells remained dispersed and extended much longer and straighter neurites. On day 2.5, the extension of neurites became evident, and approximately 50% and 75% of NGF- and mAb16-39-treated cells grew neurites, respectively (Fig. 7B,C). On days 5-7, the mAb16-39-treated cells maintained proliferation comparable to that in culture with 10% serum, and extensive interconnection of neurites was observed, whereas most of the IgG-treated cells had died. These findings indicate that ALK transmits neuritogenic signals in SK-N-SH cells. In addition, the observation that mAb16-39 suppressed cell death normally induced by serum deprivation suggests that ALK may also mediate an anti-apoptotic signal in these cells.

Sustained activation of MAP kinase is, in part, required for neuronal differentiation of PC12 cells by NGF (Marshall, 1995; York et al., 1998). The MEK inhibitor PD98059 completely suppresses neurite extension in PC12 cells overexpressing an Fc-ALK chimera (Souttou et al., 1997), suggesting that the neuritogenic activity of ALK is also MAPK pathway-dependent. Immunoblotting with anti-phospho-ERK1/2 revealed that the activation of ERK1/2 persisted after continuous exposure to mAb16-39 for 3 hours (Fig. 6A,B). Consistently, treatment of SK-N-SH cells with 50 μM



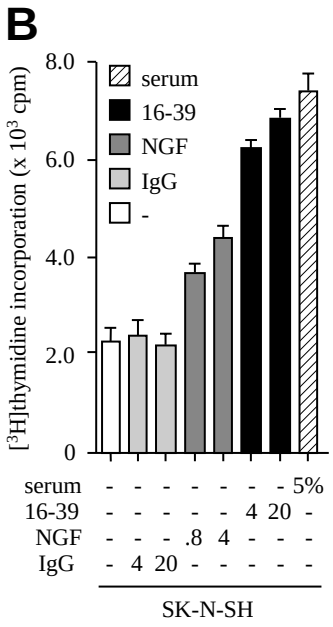


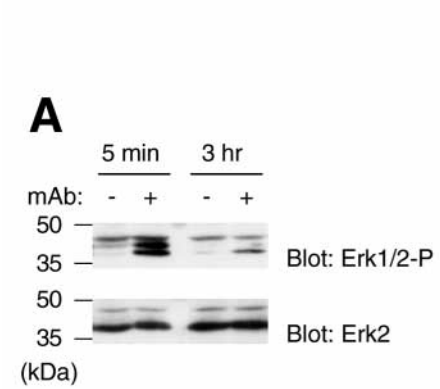
Fig. 5. Effects of mAb16-39 on DNA synthesis. Serum-deprived 2-C4, parental NIH 3T3 (A), or SK-N-SH (B) cells were cultured for 2 days in the presence of various concentrations (nM) of normal rat IgG, mAb16-39, NGF, or 5% serum as indicated. (–) in the serum column indicates 0.1% serum. [^{3}H]thymidine uptake over the last 4 hours of culture was measured and is presented as the mean±s.e.m. of three experiments performed in duplicate.

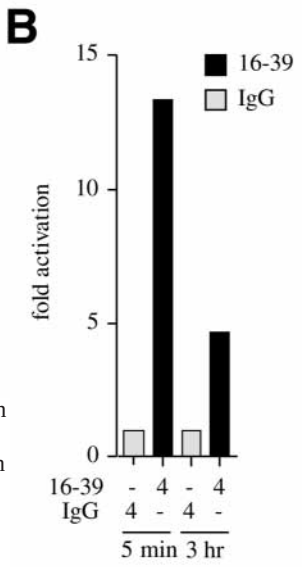
PD98059 efficiently blocked the extension of long neurites (Fig. 7D). This effect was not seen in response to treatment with 100 nM wortmannin (data not shown).

Discussion

To date, many monoclonal antibodies directed against the extracellular domain of RTKs have been created with the purpose of mimicking the agonist activity of natural ligands (Ashman et al., 1994; Fernandez-Pol, 1985; Forsayeth et al., 1987; LeSauteur et al., 1996; Prat et al., 1998; Schreiber et al., 1981; Soos et al., 1989; Spaargaren et al., 1991; Stancovski et al., 1991; Sunada et al., 1986; Xiong et al., 1992; Yarden, 1990). However, most such monoclonal antibodies promote autophosphorylation of receptors only at relatively high concentrations (10-300 nM), and few evoke downstream signaling and cellular responses (LeSauteur et al., 1996; Prat et al., 1998). In the present study, we showed that monoclonal antibody 16-39, directed against the extracellular domain of ALK, has a potent agonist activity in neuroblastoma SK-N-SH cells and fibroblast 2-C4 cells, expressing endogenous human ALK and transfected mouse ALK, respectively. In these cells, mAb16-39 not only elicited rapid and strong activation of ALK but also recruited various downstream signaling molecules to ALK and induced cellular responses in the 0.4-4 nM range. Furthermore, it is worthy of note that this is the first demonstration of an agonist mAb with neuritogenic activity.

Recently, PTN and MK have been shown to interact with the extracellular domain of ALK and proposed to be ALK ligands (Stoica et al., 2001; Stoica et al., 2002). However, this has not been confirmed by others and remains controversial. PTN activates various adaptor and effector molecules, including





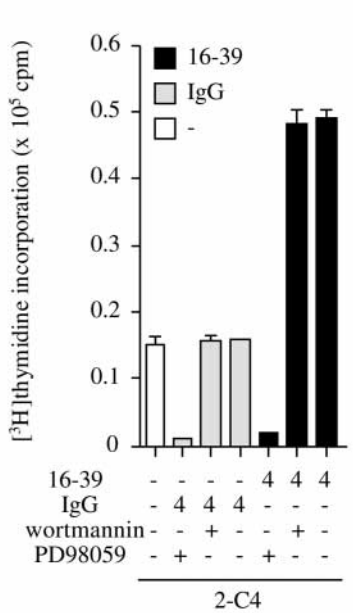
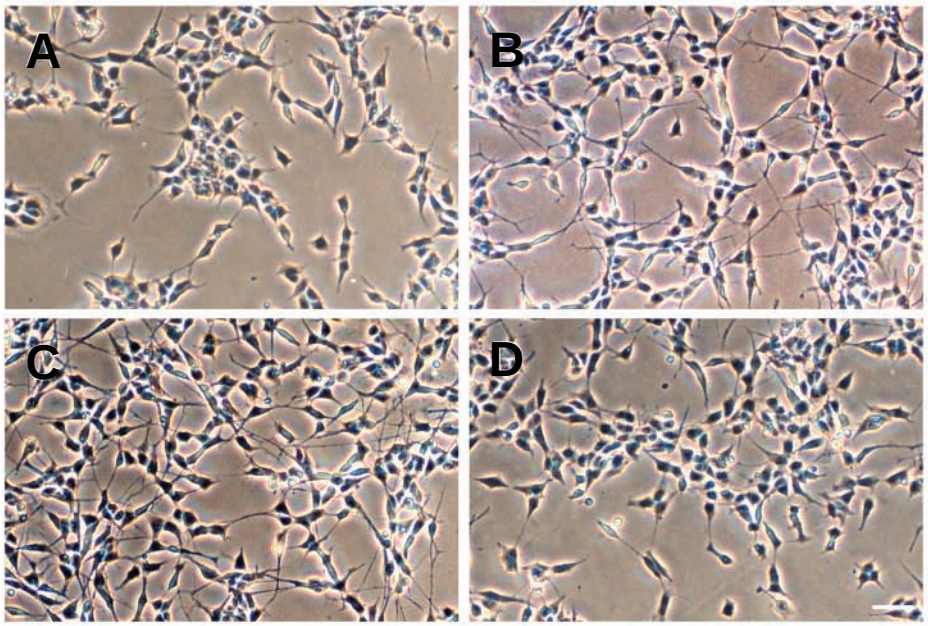


Fig. 6. Mitogenic properties of mAb16-39 are dependent on ERK activity. (A) Effect of mAb16-39 on ERK activation. 2-C4 cells were serum starved for 24 hours and treated with 4 nM normal rat IgG (–) or mAb16-39 (+) for 5 minutes or 3 hours. Equal amounts of total cell lysate were probed with phospho-specific anti-ERK1/2 antibody (ERK1/2-P). The blots were stripped and reprobed with anti-ERK antibody (ERK2). (B) Quantification of data presented in A. The intensity of the bands in the anti-phospho-ERK1/2 blot was normalized against the amounts of MAPK and expressed as fold activation over basal levels (IgG-treated). (C) Effect of the MEK inhibitor PD98059 on the mitogenic activity induced by mAb16-39. Serum-deprived 2-C4 cells were left untreated or were treated with 4 nM IgG or mAb16-39 for 2 days in the presence or absence of PD98059 (50 μM) or wortmannin (100 nM). [^{3}H]thymidine uptake assay was performed as in Fig. 5, and the data are presented as the mean±s.e.m.



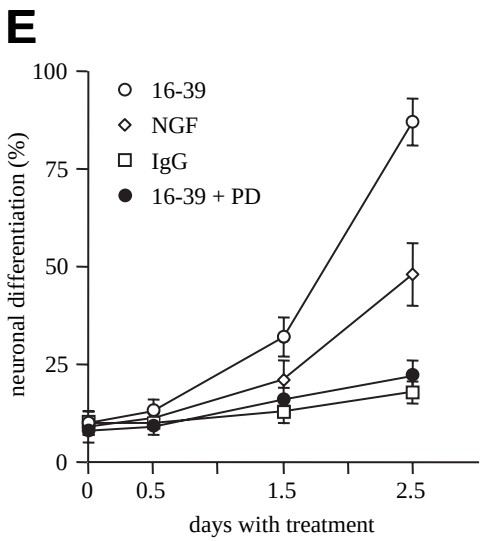


Fig. 7. mAb16-39 induces neurite outgrowth in an ERK-dependent manner. SK-N-SH cells were cultured in insulin, transferrin and selenium-supplemented media in the presence of mAb16-39 (4 nM), control normal rat IgG (4 nM), or NGF (2 nM) and photographed at day 2.5. Treatment of cells with mAb16-39 (C) resulted in extensive outgrowth of long neurites and increased cell number. Similar but less significant effects were observed in cells treated with NGF (B). Cells treated with control IgG (A) showed no significant effect on morphology. Neurite extension induced by mAb16-39 was efficiently blocked by the MEK inhibitor PD98059 (50 μM) (D). Scale bar, 50 μm. (E) Cell differentiation was scored as described in Materials and Methods and expressed as the mean±s.e.m. (%).

IRS-1, Shc, PLCγ and the p85 subunit of PI 3-kinase in SW-13/ALK cells (Stoica et al., 2001). mAb16-39-induced ALK activation also resulted in the association of these four proteins with ALK and tyrosine phosphorylation of IRS-1 and Shc. Furthermore, mAb16-39 as well as PTN and MK stimulated the MEK/ERK pathway (Owada et al., 1999; Souttou et al., 1997). Although our initial attempt to reproduce agonist activities of PTN and MK on ALK has not succeeded (data not shown), the similarity of mAb-induced signals to those of PTN (or MK) may support the physiological relevancy of these factors as ALK ligands. Cumulating evidence suggests that low-affinity receptors frequently modulate the signals from high affinity receptors (Bibel and Barde, 2000; Chao et al., 1998; Kaplan and Miller, 2000). Given a similar complexity of ligand-receptor interactions, mAb16-39 will prove useful in the analysis of the exact ALK-mediated signals by effectively dissociating them from those of low-affinity PTN receptors.

For the same reasons as detailed above, the exact cellular responses to ALK activation have been unclear. Previous studies with two different chimeric receptors have shown that the activated intracellular domain of ALK transmits differentiation and mitogenic signals in PC12 and NIH 3T3 cells, respectively (Piccinini et al., 2002; Souttou et al., 2001). Our present results show that mAb16-39 stimulates the proliferation of SK-N-SH and 2-C4 cells and promotes neurite outgrowth in SK-N-SH cells. Interestingly, whereas overexpression of an Fc-ALK chimera induced differentiation but not proliferation in PC12 cells (Souttou et al., 2001), continuous activation of ALK by mAb16-39 resulted in both proliferation and differentiation of SK-N-SH cells. This difference may be due to cell-type specificity, because NGF exerted similar effects in these cells.

Polyclonal antibodies against the extracellular domain of ALK are partially agonistic for anti-apoptosis and cell growth in NIH 3T3 cells (Bowden et al., 2002). However, we never detected the expression or tyrosine phosphorylation of endogenous ALK by western blotting and our mAb did not show any mitogenic activity in these cells (Figs 2, 3, 5). Although we do not know the exact reason for this discrepancy, one possibility is that our mAb is not effective for ALK at very low levels of expression. Alternatively, a certain population of polyclonal antibodies may have cross-reacted to other RTKs highly expressed in fibroblasts and might cause unspecific responses. Further studies are required to determine if endogenous ALK at sub-detectable levels can cause a cellular response.

The mAb-induced effects observed in neuroblastoma cells as well as fibroblasts were completely suppressed by the pharmacological inhibition of MEK activity, suggesting that the MEK/MAPK pathway plays a predominant role in transmitting mAb-induced ALK signals. The importance of the MAPK pathway is further supported by previous observations that Fc-ALK-chimera-mediated differentiation in PC12 cells, PTN-stimulated DNA synthesis in BEL cells, and the anti-apoptotic effect of PTN in NIH 3T3 cells were efficiently blocked by MEK inhibition (Bowden et al., 2002; Souttou et al., 1997; Souttou et al., 2001). In contrast, it has recently been shown that EGF treatment stimulates the proliferation of NIH 3T3 cells expressing an EGFR/ALK chimera without apparent induction of MAP kinase activity (Piccinini et al., 2002). Although the exact reason for this difference is unknown, it is conceivable that endogenous EGFR forms heterodimers with

EGFR/ALK, thereby hindering them from transmitting physiological signals. Alternatively, differences in experimental conditions such as expression levels or species of ALK may explain this difference.

NGF and insulin promote association of the p85 subunit of PI 3-kinase with several tyrosine-phosphorylated proteins ranging from 185 to 100 kDa, but the p85 subunit itself is only weakly phosphorylated on tyrosine (Hayashi et al., 1992; Ohmichi et al., 1992). Similarly, when SK-N-SH cells were stimulated with mAb16-39, p85 became associated with autophosphorylated ALK as well as with tyrosine-phosphorylated proteins of 200, 130, 95 and 70 kDa, whereas tyrosine phosphorylation of p85 itself was hardly detectable. The p85 subunit is associated with autophosphorylated RTKs directly (Ueno et al., 1997) or indirectly via SH2-containing proteins such as IRS-1 (Yamada et al., 1997), Gab1/2 (Bai et al., 2000; Holgado-Madruga et al., 1997), CRKL (Sattler et al., 1997) and c-Cbl (Soltoff and Cantley, 1996). Consistently, p85 reportedly associates with $p80^{NPM\text{-}ALK}$ indirectly through Gab2 and CRKL (Bai et al., 2000). PI 3-kinase may be thus associated with ALK and become activated in response to mAb16-39 stimulation.

The role of PI 3-kinase appears to be complicated. The PI 3-kinase/Akt pathway plays an important role in mediating anti-apoptotic effects in $p80^{NPM\text{-}ALK}$-positive hematopoietic cell lines (Bai et al., 2000; Slupianek et al., 2001). However, the ERK1/2 inhibitor UO126 but not the PI 3-kinase inhibitor LY294002 completely blocks PTN-mediated survival in serum-starved NIH 3T3 cells, thereby suggesting a predominant role of the MAPK pathway in the anti-apoptotic effects of ALK (Bowden et al., 2002). One possible explanation for this discrepancy is that $p80^{NPM\text{-}ALK}$ and wild-type ALK activate different signaling pathways due to their different cellular localizations. Alternatively, the ALK ligand may exert an anti-apoptotic effect mainly through low-affinity receptors in a MAPK-dependent manner, whereas $p80^{NPM\text{-}ALK}$ may promote survival via conventional PI 3-kinase-dependent anti-apoptotic signaling. Recent studies of NGF/TrkA/$p75^{LNTR}$ signaling indicate that $p75^{LNTR}$ generates PI 3-kinase-independent death signals, whereas concomitant signals from TrkA inhibit this effect through the MAPK pathway and promote survival via conventional PI 3-kinase-dependent anti-apoptotic signaling (Chao et al., 1998; Kaplan and Miller, 2000). A similar mechanism might be present in the ALK receptor-ligand system. As our unpublished observations suggest that our mAb also suppresses apoptosis in serum-starved SK-N-SH cells, comparative analysis with natural ALK ligand(s) and mAb16-39 may be useful in this regard.

mAb-induced activation of ALK resulted in the association of PLCγ with ALK. Tyrosine phosphorylation of PLCγ was not detected upon mAb16-39 stimulation, whereas weak phosphorylation was seen in EGFR/ALK chimera (Piccinini et al., 2002). However, the possibility that this results from the activation of endogenous EGFR cannot be excluded. PLCγ is bound to Y664 of $p80^{NPM\text{-}ALK}$ and this interaction is important for the mitogenic potency of $p80^{NPM\text{-}ALK}$ in $p80^{NPM\text{-}ALK}$-transformed Ba/F3 cells (Bai et al., 1998). However, a $p80^{NPM\text{-}ALK}$ mutant with a deleted PLCγ binding site efficiently transformed Fr 3T3 and NIH 3T3 cells (Bischof et al., 1997, and our unpublished observations). Moreover, although mouse ALK does not possess a tyrosine at the corresponding position of $p80^{NPM\text{-}ALK}$ (Y664), both human and mouse ALK associated with PLCγ at similar levels (data not shown) and promoted cell growth (Fig. 5). These observations suggest that the role of a PLCγ binding site in transmitting the ALK mitogenic signal needs to be further examined.

Although the molecular mechanism of antibody-mediated ALK activation has yet to be determined, it is likely that each Fab domain recognizes one receptor and that the bivalent antibody crosslinks two receptors, thereby leading to the formation of receptor homodimer and subsequent transactivation of the kinase. Previous observations that the Fab fragment of agonist anti-EGFR mAb 2E9 lost agonist activity and that this activity was recovered by crosslinking with secondary anti-Fab fragment antibody (Spaargaren et al., 1991) clearly support this mechanism. Thus, mAb16-39 Fab fragment may exert antagonist effects to ALK ligand(s).

In conclusion, we provide evidence that ALK transmits both mitogenic and differentiation signals in ALK-expressing neural cells via an agonist anti-ALK monoclonal antibody. Although the exact relevance of mAb16-39-induced ALK signals to those elicited by natural ligands requires further analysis, our present data support the notion that ALK functions as a membrane receptor similar to other well-characterized RTKs, and that the MAPK signaling pathway is important in mediating ALK-induced cellular responses. mAb16-39 will be useful in elucidating ALK-mediated signaling events further.

Finally, as ALK is located at the cell surface and is expressed by specific populations of normal neurons (Iwahara et al., 1997; Morris et al., 1997) and in many human neuroblastomas (Lamant et al., 2000), ALK is an excellent target candidate for antibody-based therapies. mAb16-39 may provide a basis for developing therapeutic agents, such as neurotrophic factor mimics (Maliartchouk et al., 1999), that promote survival and regeneration of neurons in neurodegenerative diseases, or immunotoxins (e.g. toxin-conjugated Fv fragment) (Forero et al., 2003) that selectively kill ALK-expressing malignant cells.

We thank J. Inoue, Y. Yamanashi, H. Nishizumi, and T. Tezuka for valuable discussions. We also thank Y. Fukui for anti-p85 monoclonal antibody. This work was supported by a grant for Advanced Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Ashman, L. K., Buhring, H. J., Aylett, G. W., Broudy, V. C. and Muller, C. (1994). Epitope mapping and functional studies with three monoclonal antibodies to the c-kit receptor tyrosine kinase, YB5.B8, 17F11, and SR-1. *J. Cell. Physiol.* **158**, 545-554.

Bai, R. Y., Dieter, P., Peschel, C., Morris, S. W. and Duyster, J. (1998). Nucleophosmin-anaplastic lymphoma kinase of large-cell anaplastic lymphoma is a constitutively active tyrosine kinase that utilizes phospholipase C-γ to mediate its mitogenicity. *Mol. Cell. Biol.* **18**, 6951-6961.

Bai, R. Y., Ouyang, T., Miething, C., Morris, S. W., Peschel, C. and Duyster, J. (2000). Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. *Blood* **96**, 4319-4327.

Ben-Neriah, Y. and Bauskin, A. R. (1988). Leukocytes express a novel gene encoding a putative transmembrane protein-kinase devoid of an extracellular domain. *Nature* **333**, 672-676.

Bibel, M. and Barde, Y. A. (2000). Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**, 2919-2937.

Bischof, D., Pulford, K., Mason, D. Y. and Morris, S. W. (1997). Role of the nucleophosmin (NPM) portion of the non-Hodgkin's lymphoma-associated NPM-anaplastic lymphoma kinase fusion protein in oncogenesis. *Mol. Cell. Biol.* **17**, 2312-2325.

Borer, R. A., Lehner, C. F., Eppenberger, H. M. and Nigg, E. A. (1989). Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell* **56**, 379-390.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H. and Yancopoulos, G. D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**, 663-675.

Bowden, E. T., Stoica, G. E. and Wellstein, A. (2002). Anti-apoptotic signaling of pleiotrophin through its receptor, anaplastic lymphoma kinase. *J. Biol. Chem.* **277**, 35862-35868.

Burchill, S. A., Berry, P. A. and Lewis, I. J. (1995). Activation of $p21^{ras}$ by nerve growth factor in neuroblastoma cells. *J. Neurol. Sci.* **133**, 3-10.

Chao, M., Casaccia-Bonnefil, P., Carter, B., Chittka, A., Kong, H. and Yoon, S. O. (1998). Neurotrophin receptors: mediators of life and death. *Brain Res. Rev.* **26**, 295-301.

Fernandez-Pol, J. A. (1985). Epidermal growth factor receptor of A431 cells. *J. Biol. Chem.* **260**, 5003-5011.

Forero, A. and LoBuglio, A. F. (2003). History of antibody therapy for non-Hodgkin's lymphoma. *Semin. Oncol.* **30**, 1-5.

Forsayeth, J. R., Montemurro, A., Maddux, B. A., DePirro, R. and Goldfine, I. D. (1987). Effect of monoclonal antibodies on human insulin receptor autophosphorylation, negative cooperativity, and down-regulation. *J. Biol. Chem.* **262**, 4134-4140.

Fujimoto, J., Shiota, M., Iwahara, T., Seki, N., Satoh, H., Mori, S. and Yamamoto, T. (1996). Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). *Proc. Natl. Acad. Sci. USA* **93**, 4181-4186.

Grewal, S. S., York, R. D. and Stork, P. J. (1999). Extracellular-signal-regulated kinase signaling in neurons. *Curr. Opin. Neurobiol.* **9**, 544-553.

Hayashi, H., Kamohara, S., Nishioka, Y., Kanai, F., Miyake, N., Fukui, Y., Shibasaki, F., Takenawa, T. and Ebina, Y. (1992). Insulin treatment stimulates the tyrosine phosphorylation of the α-type 85-kDa subunit of phosphatidylinositol 3-kinase *in vivo*. *J. Biol. Chem.* **267**, 22575-22580.

Holgado-Madruga, M., Moscatello, D. K., Emlet, D. R., Dieterich, R. and Wong, A. J. (1997). Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by neve growth factor. *Proc. Natl. Acad. Sci. USA* **94**, 12419-12424.

Iwahara, T., Fujimoto, J., Wen, D., Cupples, R., Bucay, N., Arakawa, T., Mori, S., Ratzkin, B. and Yamamoto, T. (1997). Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* **14**, 439-449.

Janet, T., Ludecke, G., Otten, U. and Unsicker, K. (1995). Heterogeneity of human neuroblastoma cell lines in their proliferative responses to basic FGF, NGF, and EGF: correlation with expression of growth factors and growth factor receptors. *J. Neurosci. Res.* **40**, 707-715.

Kaplan, D. R. and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381-391.

Kazlauskas, A. (1994). Receptor tyrosine kinases and their targets. *Curr. Opin. Genet. Dev.* **4**, 5-14.

Kojima, T., Katsumi, A., Yamazaki, T., Muramatsu, T., Nagasaka, T., Ohsumi, K. and Saito, H. (1996). Human ryudocan from endothelium-like cells binds basic fibroblast growth factor, midkine, and tissue factor pathway inhibitor. *J. Biol. Chem.* **271**, 5914-5920.

Kotani, M., Yamamura, Y., Tamatani, T., Kitamura, F. and Miyasaka, M. (1993). Generation and characterization of monoclonal antibodies against rabbit CD4, CD5 and CD11a antigens. *J. Immunol. Methods* **157**, 241-252.

Krolewski, J. J. and Dalla-Favera, R. (1991). The ltk gene encodes a novel receptor-type protein tyrosine kinase. *EMBO J.* **10**, 2911-2919.

Lamant, L., Pulford, K., Bischof, D., Morris, S. W., Mason, D. Y., Delsol, G. and Mariame, B. (2000). Expression of the ALK tyrosine kinase gene in neuroblastoma. *Am. J. Pathol.* **156**, 1711-1721.

LeSauteur, L., Maliartchouk, S., le Jeune, H., Quirion, R. and Saragovi, H. U. (1996). Potent human p140-TrkA agonists derived from an anti-receptor monoclonal antibody. *J. Neurosci.* **16**, 1308-1316.

Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A. et al. (1999). Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* **4**, 1029-1040.

Maeda, N., Nishiwaki, T., Shintani, T., Hamanaka, H. and Noda, M. (1996). 6B4 proteoglycan/phosphacan, an extracellular variant of receptor-like protein-tyrosine phosphatase ζ/RPTPβ, binds pleiotrophin/heparin-binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* **271**, 21446-21452.

Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T. and Noda, M. (1999). A receptor-like protein-tyrosine phosphatase PTPζ/RPTPβ binds a heparin-binding growth factor midkine. *J. Biol. Chem.* **274**, 12474-12479.

Maliartchouk, S., Feng, Y., Ivanisevic, L., Debeir, T., Cuello, A. C., Burgess, K. and Saragovi, H. U. (1999). A designed peptidomimetic agonistic ligand of TrkA nerve growth factor receptors. *Mol. Pharmacol.* **57**, 385-391.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Mitsiadis, T. A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalkanen, M. and Thesleff, I. (1995). Expression of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* **121**, 37-51.

Morris, S. W., Kirstein, M. N., Valentine, M. B., Dittmer, K. G., Shapiro, D. N., Saltman, D. L. and Look, A. T. (1994). Fusion of a kinase gene, *ALK*, to a nucleolar protein gene, *NPM*, in non-Hodgkin's lymphoma. *Science* **263**, 1281-1284.

Morris, S. W., Naeve, C., Mathew, P., James, P. L., Kirstein, M. N., Cui, X. and Witte, D. P. (1997). *ALK*, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* **14**, 2175-2188.

Muramatsu, T. (2002). Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. *J. Biochem. (Tokyo)* **132**, 359-371.

Ohmichi, M., Decker, S. J. and Saltiel, A. R. (1992). Activation of phosphatidylinositol-3 kinase by nerve growth factor involves indirect coupling of the *trk* proto-oncogene with src homology 2 domains. *Neuron* **9**, 769-777.

Okuda, M., Horn, H. F., Tarapore, P., Tokuyama, Y., Smulian, A. G., Chan, P. K., Knudsen, E. S., Hofmann, I. A., Snyder, J. D., Bove, K. E. et al. (2000). Nucleophosmin/B23 is a target of Cdk2/Cyclin E in centrosome duplication. *Cell* **103**, 127-140.

Owada, K., Sanjo, N., Kobayashi, T., Mizusawa, H., Muramatsu, H., Muramatsu, T. and Michikawa, M. (1999). Midkine inhibits caspase-dependent apoptosis via the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase in cultured neurons. *J. Neurochem.* **73**, 2084-2092.

Piccinini, G., Bacchiocchi, R., Serresi, M., Vivani, C., Rossetti, S., Gennaretti, C., Carbonari, D. and Fazioli, F. (2002). A ligand-inducible epidermal growth factor receptor/anaplastic lymphoma kinase chimera promotes mitogenesis and transforming properties in 3T3 cells. *J. Biol. Chem.* **277**, 22231-22239.

Prat, M., Crepaldi, T., Pennacchietti, S., Bussolino, F. and Comoglio, P. M. (1998). Agonistic monoclonal antibodies against the Met receptor dissect the biological responses to HGF. *J. Cell Sci.* **111**, 237-247.

Raulo, E., Chernousov, M. A., Carey, D. J., Nolo, R. and Rauvala, H. (1994). Isolation of a neuronal cell surface receptor of heparin binding growth-associated molecule (HB-GAM). *J. Biol. Chem.* **269**, 12999-13004.

Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Pisick, E., Prasad, K. V. and Griffin, J. D. (1997). Steel factor induces tyrosine phosphorylation of CRKL and binding of CRKL to a complex containing c-Kit, phosphatidylinositol 3-kinase, and $p120^{CBL}$. *J. Biol. Chem.* **272**, 10248-10253.

Schreiber, A. B., Lax, I., Yarden, Y., Eshhar, Z. and Schlessinger, J. (1981). Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor. *Proc. Natl. Acad. Sci. USA* **78**, 7535-7539.

Sclessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225.

Shiota, M., Fujimoto, J., Semba, T., Satoh, H., Yamamoto, T. and Mori, S. (1994). Hyperphosphorylation of a novel 80 kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. *Oncogene* **9**, 1567-1574.

Shiota, M., Nakamura, S., Ichinohasama, R., Abe, M., Akagi, T., Takeshita, M., Mori, N., Fujimoto, J., Miyauchi, J., Mikata, A. et al. (1995). Anaplastic large cell lymphomas expressing the novel chimeric protein $p80^{NPM/ALK}$: a distinct clinicopathologic entity. *Blood* **86**, 1954-1960.

Slupianek, A., Nieborowska-Skorska, M., Hoser, G., Morrione, A., Majewski, M., Xue, L., Morris, S. W., Wasik, M. A. and Skorski, T. (2001). Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. *Cancer Res.* **61**, 2194-2199.

Soltoff, S. P. and Cantley, L. C. (1996). p120^{cbl} is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. *J. Biol. Chem.* **271**, 563-567.

Soos, M. A., O'Brien, R. M., Brindle, N. P., Stigter, J. M., Okamoto, A. K., Whittaker, J. and Siddle, K. (1989). Monoclonal antibodies to the insulin receptor mimic metabolic effects of insulin but do not stimulate receptor autophosphorylation in transfected NIH 3T3 fibroblasts. *Proc. Natl. Acad. Sci. USA* **86**, 5217-5221.

Souttou, B., Ahmad, S., Riegel, A. T. and Wellstein, A. (1997). Signal transduction pathways involved in the mtogenic activity of pleiotrophin. *J. Biol. Chem.* **272**, 19588-19593.

Souttou, B., Carvalho, N. B., Raulais, D. and Vigny, M. (2001). Activation of anaplastic lymphoma kinase receptor tyrosine kinase induces neuronal differentiation through the mitogen-activated protein kinase pathway. *J. Biol. Chem.* **276**, 9526-9531.

Spaargaren, M., Defize, L. H., Boonstra, J. and de Laat, S. W. (1991). Antibody-induced dimerization activates the epidermal growth factor receptor tyrosine kinase. *J. Biol. Chem.* **266**, 1733-1739.

Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y. and Sela, M. (1991). Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. *Proc. Natl. Acad. Sci. USA* **88**, 8691-8695.

Stoica, G. E., Kuo, A., Aigner, A., Sunitha, I., Souttou, B., Malerczyk, C., Caughey, D. J., Wen, D., Karavanov, A., Riegel, A. T. et al. (2001). Identification of anaplastic lymphoma kinase as a receptor for the growth factor pleiotrophin. *J. Biol. Chem.* **276**, 16772-16779.

Stoica, G. E., Kuo, A., Powers, C., Bowden, E. T., Sale, E. B., Riegel, A. T. and Wellstein, A. (2002). Midkine binds to anaplastic lymphoma kinase (ALK) and acts as a grwoth factor for different cell types. *J. Biol. Chem.* **277**, 35990-35998.

Sunada, H., Magun, B. E., Mendelsohn, J. and MacLeod, C. L. (1986). Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. *Proc. Natl. Acad. Sci. USA* **83**, 3825-3829.

Ueno, H., Honda, H., Nakamoto, T., Yamagata, T., Sasaki, K., Miyagawa, K., Mitani, K., Yazaki, Y. and Hirai, H. (1997). The phosphatidylinositol 3′ kinase pathway is required for the survival signal of leukocyte tyrosine kinase. *Oncogene* **14**, 3067-3072.

Xiong, L., Kasuya, J., Li, S. L., Kato, J. and Fujita-Yamaguchi, Y. (1992). Growth-stimulatory monoclonal antibodies against human insulin-like growth factor I receptor. *Proc. Natl. Acad. Sci. USA* **89**, 5356-5360.

Yamada, M., Ohnishi, H., Sano, S., Nakatani, A., Ikeuchi, T. and Hatanaka, H. (1997). Insulin receptor substrate (IRS)-1 and IRS-2 are tyrosine-phosphorylated and associated with phosphatidylinositol 3-kinase in response to brain-derived neurotrophic factor in cultured cerebral cortical neurons. *J. Biol. Chem.* **272**, 30334-30339.

Yarden, Y. (1990). Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constitutively active. *Proc. Natl. Acad. Sci. USA* **87**, 2569-2573.

Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A. and Baron, R. (1999). Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J. Biol. Chem.* **274**, 31707-31712.

York, R. D., Yao, H., Dillon, T., Ellig, C. L., Eckert, S. P., McCleskey, E. W. and Stork, P. J. (1998). Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622-626.