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ORIGINAL ARTICLE

The constitutive activity of the ALK mutated at positions F1174 or R1275 impairs receptor trafficking

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Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK), which is transiently expressed during development of the central and peripheral nervous system. ALK has been recently identified as a major neuroblastoma predisposition gene and activating mutations have also been identified in a subset of sporadic neuroblastoma tumors. Two hot spots of ALK mutations have been observed at positions F1174 and R1275. Here, we studied stably transfected cell lines expressing wild-type or F1174L- or R1275Q-mutated ALK in parallel with a neuroblastoma cell line (CLB-GE) in which the allele mutated at position F1174 is amplified. We observed that the mutated ALK variants were essentially intracellular and were largely retained in the reticulum/Golgi compartments. This localization was corroborated by a defect of *N*-linked glycosylation. Although the mutated receptors exhibited a constitutive activation, the minor pool of receptor addressed to the plasma membrane was much more tyrosine phosphorylated than the intracellular pool. The use of antagonist monoclonal antibodies suggested that the constitutive activity of the mutated receptors did not require the dimerization of the receptor, whereas adequate dimerization triggered by agonist monoclonal antibodies increased this activity. Finally, kinase inactivation of the mutated receptors restored maturation and cell-surface localization. Our results show that constitutive activation of ALK results in its impaired maturation and intracellular retention. Furthermore, they provide a rationale for the potential use of kinase inhibitors and antibodies in ALK-dependent tumors.
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Introduction

The intracellular domain of Anaplastic Lymphoma Kinase (ALK) has been initially identified in some anaplastic large cell lymphomas through the characterization of fusion proteins resulting from chromosomal translocations (for review see (Palmer *et al*., 2009)). Full-length ALK receptor tyrosine kinase (RTKs) have been described in human, mouse, *Drosophila* and *Caenorhabditis elegans* (Iwahara *et al*., 1997; Morris *et al*., 1997; Loren *et al*., 2001; Reiner *et al*., 2008). The distribution of ALK transcripts strongly suggests that this receptor could have an important role in the normal development and function of the nervous system (Iwahara *et al*., 1997; Morris *et al*., 1997; Vernersson *et al*., 2006). However, in vertebrates the biological functions of this receptor are largely unknown.

Recently, the full-length receptor ALK has been characterized as a neuroblastoma predisposition gene and was shown to be also involved in a subset of sporadic neuroblastoma cases (Caren *et al*., 2008; Chen *et al*., 2008; George *et al*., 2008; Janoueix-Lerosey *et al*., 2008; Mosse *et al*., 2008). Indeed, activating ALK mutations were identified in both familiar and sporadic cases of neuroblastoma with two hot spots occurring at positions F1174 and R1275 (Janoueix-Lerosey *et al*., 2010).

The neuroblastoma cell lines in which ALK point mutations have been identified are heterozygous and therefore express both the wild-type and the mutated receptor. In this study, we investigated the status of stably transfected NIH3T3 cell lines expressing the wild-type ALK (ALK-WT) receptor or the F1174L-mutated ALK (ALK-F1174L) or the R1275Q-mutated ALK (ALK-R1275Q). In parallel, we investigated a neuroblastoma cell line (CLB-GE) that carries an amplified allele coding for F1174V-mutated ALK (Janoueix-Lerosey *et al*., 2008). To be able to clarify the ALK biological roles in the absence of an established ALK cognate ligand(s) in vertebrates (for review see (Palmer *et al*., 2009)), we employed monoclonal antibodies (mAb), which are able to stimulate or inhibit the ALK-WT activity (Moog-Lutz *et al*., 2005; Mathivet *et al*., 2007; Yang *et al*., 2007).

We first observed that the ALK-WT was present at the plasma membrane but also exhibited an intracellular

localization. In contrast, mutated forms of ALK were essentially retained intracellularly in the reticulum/Golgi compartment, which is characteristic of misfolded proteins. Kinase inactivation restored receptor maturation and plasma membrane addressing. Importantly, this cellular localization largely influenced the constitutive activity of the mutated receptors.

Results

The wild-type ALK receptor is partially addressed to the plasma membrane

In the developing brain (Iwahara *et al*., 1997; Morris *et al*., 1997), in neuroblastoma cell lines endogenously expressing ALK (Miyake *et al*., 2002; Motegi *et al*., 2004; Mathivet *et al*., 2007; George *et al*., 2008) or in cells expressing this receptor after transfection (Moog-Lutz *et al*., 2005), ALK is expressed as the 220 kDa full-length receptor and a shorter form of 140 kDa (Figure 1). We previously established that the 140 kDa species results from an extracellular cleavage of the 220 kDa form (Moog-Lutz *et al*., 2005). The protein band at 220 kDa often appeared as a doublet of two closed ALK molecules, the nature of which has not yet been determined (Mourali *et al*., 2006; Mathivet *et al*., 2007). ALK-WT stably expressed in NIH 3T3 cell lines (named 3T3/WT) exhibited almost no basal tyrosine phosphorylation (Figure 1a). Addition of agonist mAb46 resulted in a strong phosphorylation of the upper band of 220 kDa doublet and of the 140 kDa form, but not of the 220 kDa lower band (Figure 1a). As previously established, antagonist mAb30 strongly inhibited the activation triggered by mAb46. MAb30 dimerizes and lockes two ALK receptor molecules in a conformational state in which no trans-activation of the tyrosine kinase domain can occur (Moog-Lutz *et al*., 2005). The phosphorylation of the lower band of the 220 kDa doublet did not exhibit any change upon agonist and/or antagonist mAbs treatments. It suggested that this band was not accessible to the mAbs, possibly because of an intracellular localization. We performed cell-surface protein biotinylation procedures on 3T3/WT and demonstrated that the lower band of the 220 kDa doublet was indeed intracellular as it was stained by ALK antibodies but not by biotin. On contrary, both the upper 220 kDa band and the 140 kDa form were stained by streptavidin, indicating localization at the plasma membrane (Figure 1b).

Constitutive activation and cellular localization of the ALK-F1174L mutant

We next employed NIH3T3 cell lines stably transfected with ALK-F1174L (named 3T3/F1174L). In these cell

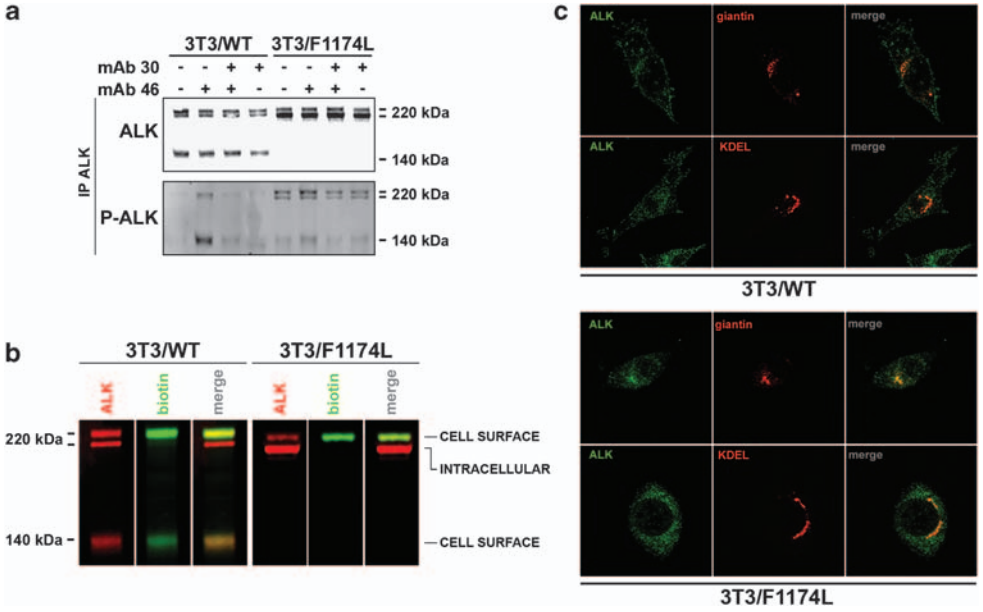

Figure 1 Constitutive activity and cellular localization of wild-type and F1174L-mutated ALK. (**a**) 3T3/WT ALK and 3T3/F1174L cells were untreated (lane 1), treated with 6 nM of agonist mAb46 for 20 min (lane 2), pretreated with 48 nM of mAb30 for 30 min and then with 6 nM of mAb46 for 20 min (lane 3) or treated with 48 nM of antagonist mAb30 for 50 min (lane 4). Cell extracts were prepared by lysing the cells in a RIPA buffer and subjected to immunoprecipitation. Immunoprecipitates were subjected to immunobloting using anti-phosphotyrosine (4G10) or polyclonal anti-ALK (REAB). (**b**) 3T3/WT and 3T3/F1174L cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-biotin for 30 min on ice, and then washed with cold PBS-Glycin 0.1 mM. ALK were immunoprecipitated from cell lysates and submitted to western blot analysis. Total ALK were detected with polyclonal anti-ALK (Zymed) and secondary anti-rabbit IgG coupled to IRdye700 (in red), biotinylated ALK (cell surface receptor) with streptavidin coupled with IRdye800 (in green). (**c**) Immunofluoresence confocal microscopy analysis of subcellular localization ALK in 3T3/WT and 3T3/F1174L. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 before staining. Cells were immunostained, respectively, for ALK (mAb46/15, in green) (upper panel) and Golgi apparatus (polyclonal anti-Giantin, in red) or ALK (polyclonal anti-ALK Zymed, in green) and endoplasmic reticulum (monoclonal anti-KDEL, in red) (lower panel).

lines the protein band at 220 kDa also appeared as a doublet of ALK, but compared with cell lines expressing the WT receptor the lower band was much more intense than the upper band (Figure 1a). With respect to the data obtained with the WT receptor (see above), this suggests that the ALK F1174L mutant is largely intracellularly localized. For 3T3/F1174L, cell-surface biotinylation of plasma membrane proteins indeed confirmed that the lower band of 220 kDa doublet was intracellular (Figure 1b). It should be added that the 140 kDa form was hardly discernable in the extracts of the mutated cell lines. This result was expected as the mutated forms of ALK are largely intracellular and the 140 kDa species results from an extracellular cleavage of the full-length 220 kDa form.

When compared with ALK-WT, the mutated receptor exhibited a constitutive activation but intriguingly the minor pool of receptors addressed to the plasma membrane (upper 220 kDa band) was much more tyrosine phosphorylated than the intracellular pool (lower 220 kDA band, Figure 1a). Agonist mAb46 treatment increased the phosphorylation both of the upper band of the 220 doublet and of the 140 kDa form, the forms present at the plasma membrane. The antagonist mAb30 indeed reduced the degree of induced phosphorylation by mAb46 of ALK-F1174L. However, this mAb30 had no effect on the basal phosphorylation of the upper band of 220 kDa doublet (the sole form accessible to the mAbs) suggesting that the constitutive activity of the ALK-F1174L receptor does not require ligand-induced dimerization (Figure 1a). In contrast, agonist mAb46 increased the basal phosphorylation of ALK-F1174L (Figure 1a) suggesting that this constitutive activation is incomplete and can be further increased by the dimerization triggered in presence of agonist mAbs.

Finally, confocal microscopy after immunofluorescence staining experiments revealed that ALK-WT in 3T3/WT cells was partially located at the plasma membrane but also exhibited an intracellular localization. In agreement with biotinylation experiments, ALK-F1174L in these cells was essentially intracellular. Both intracellular ALK-WT and ALK-F1174L largely colocalized with markers of the reticulum and Golgi apparatus (Figure 1c).

The lower band of 220 kDa doublet is partially glycosylated

The fact that the phosphorylation of the lower band of the 220 kDa doublet was not modulated by mAb treatments suggested that it was not accessible to the mAbs because of an intracellular localization. We therefore hypothesized that the difference between the two forms of the 220 kDa doublet may correspond to different degrees of glycosylation. The processing of *N*-linked glycosylation of transmembrane-bound proteins occurs in several steps (Kornfeld and Kornfeld, 1985) and full glycoprotein maturation is necessary for plasma membrane targeting (Helenius and Aebi, 2004). In the endoplasmic reticulum there is first an acquisition

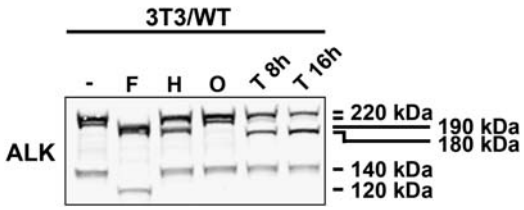

Figure 2 The lower band of the 220 kDa doublet of wild-type ALK is Endo-H-sensitive and partially processed. ALK immunoprecipitates from 3T3/WT cell extract were incubated with N-glycosidase F (lane 2, F) or endoglycosidase H (lane 3, H) or O-glycosidase (lane 4, O). Deglycosilation profiles were compared with nontreated immunoprecipitate (lane 1) and with immunoprecipitates of 3T3/WT cells treated with 10 μg/ml tunikamycin for 8 and 16 h (lanes 5 and 6, T) by western blot analysis using polyclonal anti-ALK (REAB).

of *N*-linked oligosaccharides, followed by the removal of terminal glucose and mannose residues. The partially processed polypeptide is then transported to Golgi apparatus, in which mannose residues are further trimmed and *N*-acetylglucosamine, galactose and sialic acid residues are sequentially added. This glycosylation process can be partially revealed using endoglycosidase H, which cleaves high mannose-containing immature *N*-linked carbohydrates and endoglycosidase F, which cleaves both the immature *N*-linked carbohydrates and complex-type oligosaccharides from glycoproteins. In response to endoglycosidase F treatment, molecular mass of ALK-WT decreased for the 220 kDa doublet to about 190 kDa and for the 140 kDa form to 120 kDa. In contrast, only the lower band of the 220 kDa doublet was sensitive to endoglycosidase H treatment, indicating that this band corresponds to a partially glycosylated form of ALK likely retained in the endoplasmic reticulum (Figure 2). Endoglycosidase O had no effect. We also treated the same cell line with tunikamycin (a classical inhibitor of asparagine-linked glycosylation). A band of about 180 kDa corresponding to the newly synthesized completely nonglycosylated receptor was detected.

Treatment of ALK immunoprecipitated from 3T3/F1174L cell extract with endoglycosidase F, H or O led to identical conclusions (data not shown and see below).

The constitutive activity of ALK-F1174L controls its intracellular localization

The retention in the reticulum is characteristic of misfolded proteins (Helenius and Aebi, 2004). We therefore investigated whether the constitutive activity of ALK-F1174L could induce its retention in the reticulum and Golgi apparatus. To address this question, we used the selective ALK tyrosine kinase inhibitor NVP-TAE684 (TAE) (Galkin *et al*., 2007). We reasoned that inhibition of the constitutive activity of ALK-F1174L might modify its cellular localization. First, we investigated the effect of TAE on the activation of the wild-type form of ALK. In 3T3/WT cells there was no basal activation of either ALK or Erk 1/2

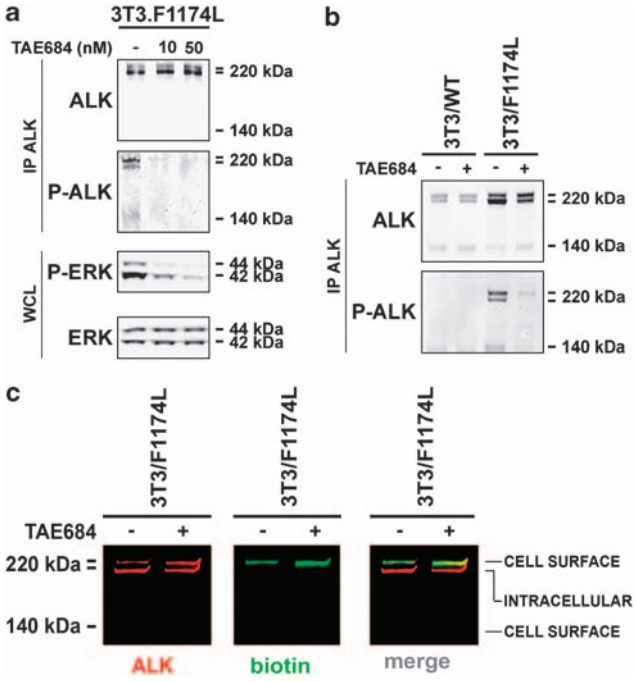

Figure 3 Effects of tyrosine kinase inhibitor NVP-TAE684 on F1174L-mutated ALK. (**a**) 3T3/F1174L cells were nontreated (lane 1) or treated with 10 nM (lane 2) or with 50 nM (lane 3) of NVP-TAE684 (TAE) for 1 h. Cells extracts were western-blotted with antibodies against phospho-ERK1/2 and total ERK1/2, and ALK immunoprecipitates were subjected to immunoblotting with anti-phosphotyrosine antibody (4G10) and anti-ALK (REAB). (**b**) 3T3/WT (lanes 1–2) and 3T3/F1174L (lanes 3–4) cells were nontreated (lanes 1–3) or treated with 50 nM of TAE for 24 h (lanes 2–4). ALK immunoprecipitates from cell extracts were analyzed by western blot with anti-ALK antibody (REAB) and anti-phosphotyrosine antibody (4G10). (**c**) 3T3/F1174L cells were incubated (+) or not (−) with 50 nM of TAE for 24 h. They were then subjected to biotinylation procedure as described in Figure 1b. ALK immunoprecipitates were submitted to western blot analysis. Total (in red) and biotinylated ALK (cell surface receptor, in green) were detected as described in Figure 1b.

kinases (Figure 1a). Upon stimulation with the mAb46, the wild-type ALK receptor is phosphorylated and mediates phosphorylation of downstream targets, such as Erk 1/2 (Figure 1a). Treatment with TAE at concentration of 50 nM before stimulation inhibits the induced phosphorylation of the 220 and 140 kDa forms of wild-type ALK receptor (Supplementary Figure S1).

In NIH 3T3 cells, basal phosphorylation of ALK-F1174L was completely inhibited after 1 h incubation in presence of 10 nM of TAE (Figure 3a). The TAE inhibition occurred rapidly (less than 15 min) and persisted for at least 24 h without any further addition of the inhibitor (Figure 3b). Further, inhibition of the ALK-F1174L by TAE correlates with a decreasing phosphorylation of the Erk kinases (Figure 3a). Thus, the constitutive activation mediated by the ALK F1174L receptor, which mediates the phosphorylation of downstream target, such as the Erk kinase pathway, is likely to be important for neuroblastoma progression.

After a 24 h treatment with 50 nM TAE, the pattern of expression of the ALK-WT was unchanged. In contrast for ALK-F1174L, the TAE treatment induced an important diminution of the lower band of 220 kDa doublet leading to a pattern of expression similar to that of the WT receptor (Figure 3b). Therefore, kinase inhibition restored receptor maturation and cell-surface localization as demonstrated by biotinylation experiments (Figure 3c).

Constitutive activity and intracellular localization of ALK-F1174V in CLB-GE cells

The allele mutated at position F1174 (F1174V) is amplified (Janoueix-Lerosey *et al*., 2008) in the CLB-GE neuroblatoma cell line. This cell line provided us with the opportunity to analyze the properties of this mutated receptor with endogenous expression and highly predominant with respect to the WT form. In this cell line, ALK was essentially present as the lower intracellular band of the 220 kDa doublet (Figure 4a). Biotinylation experiments (Supplementary Figure S2A) further confirmed that this band was intracellular. Treatments with endoglycosidase H and F confirmed that this band indeed correspond to a glycoprotein containing immature *N*-linked carbohydrates (Supplementary Figure S2B). ALK-F1174V exhibited a constitutive activation (comparison with the IMR-32 neuroblastoma cell line that expressed only ALK-WT) but again the minor pools of receptors addressed to the plasma membrane were much more tyrosine phosphorylated than the intracellular pool (Figure 4a). The antagonist mAb30 had no effect on the basal phosphorylation of the upper band of 220 kDa doublet, whereas mAb46 increased this basal phosphorylation (Figure 4a). Again these results suggested that the constitutive activity of the mutated receptor did not require its dimerization, whereas it could be further increased by an adequate dimerization triggered in the presence of agonist mAbs. Immunocytochemical experiments also indicated that the ALK receptor in the CLB-GE cells was essentially intracellular (Figure 4b).

Constitutive activation and cellular localization of the ALK-R1275Q mutant

We next investigated the status of ALK mutated at position R1275. For that purpose we used three NIH3T3 cell lines stably transfected with ALK-R1275Q (named 3T3/R1275Q). The properties of ALK-R1275Q receptor were highly similar but not identical to those found and described for the ALK F1174L mutant. First, concerning the relative ratio of the two bands of the 220 kDa doublet, the lower band was much more intense than the upper band (Figure 5a). Note, however, that, compared with the ALK F1174L mutant, the upper band of the 220 kDa doublet was more expressed (compare Figures 1a and 5a) suggesting that the ALK-R1275Q receptor is proportionally more addressed to the plasma membrane than the other mutant. This hypothesis was supported by the fact that the 140 kDa form resulting from an extracellular cleavage of the full-length receptor was clearly discernable (Figure 5a). Yet cell-surface biotinylation of plasma membrane proteins indeed confirmed that the lower

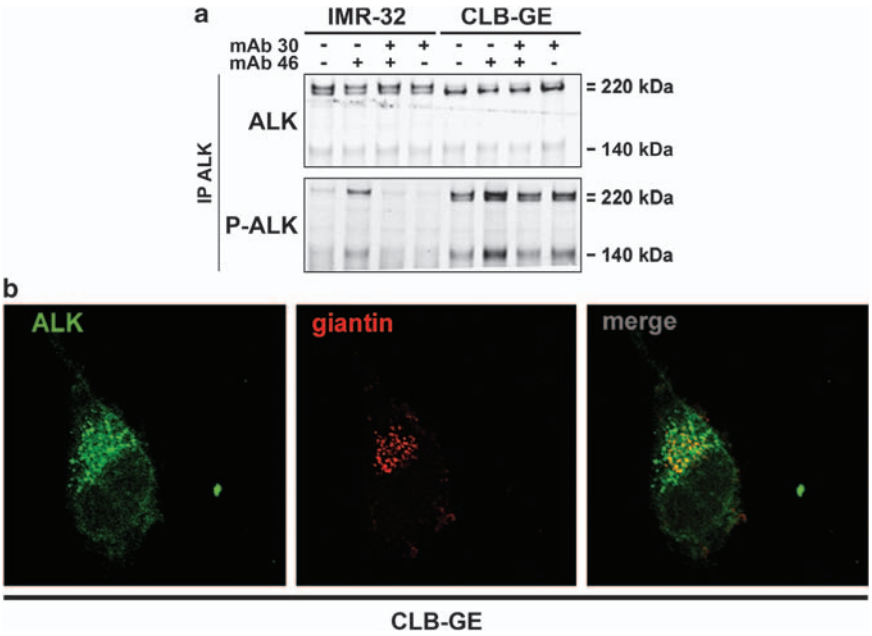

Figure 4 Intracellular retention of mutated F1174V ALK in neuroblastoma cell line CLB-GE. (**a**) IMR-32 (lane 1–4) or CLB-GE cells (lanes 5–8) were untreated (lane 1–5), treated with 48 nM of antagonist mAb30 for 50 min (lane 2–6), treated with 6 nM of agonist mAb46 for 20 min (lane 3–7) or pretreated with 48 nM of mAb30 for 30 min and then treated with 6 nM of mAb46 for 20 min (lanes 4–8). Cells extracts were subjected to immunoprecipitation and immunoprecipitates were submitted to western blot analysis using polyclonal anti-ALK (REAB) or anti-phosphotyrosine (4G10). Important note: mutated ALK is amplified in the CLB-GE cell line. Thus, to allow a comparison of the phosphorylation profiles between the two cell lines, similar amounts of immunoprecipitates ALK were loaded. This blot is not representative of the level of expression of the receptor between the two cell lines. (**b**) Immunofluoresence confocal microscopy analysis of subcellular localization ALK in CLB-GE cells. Cells were fixed with paraformaldehyde and permeabelized with Triton X-100 before staining. Cells were immunostained, respectively, for ALK (mAb46/15, in green) and Golgi apparatus (polyclonal anti-Giantin, in red).

band of 220 kDa doublet was intracellular (see below). When compared with ALK-WT, the mutated receptor exhibited a constitutive activation, but again the minor pool of receptors addressed to the plasma membrane (upper 220 kDa band) was much more tyrosine phosphorylated than the intracellular pool (lower 220 kDa band, Figure 5a). In fact the phosphorylation pattern of the two bands of the 220 kDa doublet was a mirror image of the protein pattern. MAb30 had no effect on the basal phosphorylation of the upper band of 220 kDa doublet but reduced the degree of induced phosphorylation by mAb46 (Figure 5a). These data again suggested that the constitutive activity of the ALK-R1275Q receptor does not require dimerization, but that this constitutive activation can be further increased by the adequate dimerization triggered by the agonist mAb.

Finally, we investigated whether kinase inhibition restored ALK-R1275Q receptor maturation and cell-surface localization. TAE indeed inhibited the constitutive activity of the ALK-R1275Q (Figure 5b) but this mutant was less sensitive to TAE than the ALK-F1174L mutant (Figure 3a). After 1 h treatment complete, inhibition of the ALK-R1275Q required 50 nM TAE, whereas this inhibition was achieved with only 10 nM TAE for the ALK-F1174L mutant. Such a difference of TAE sensitivity between the two mutants has been reported by others (George *et al*., 2008). This inhibition persisted after a 24 h treatment with 50 nM TAE without any further addition of the inhibitor (Figure 5c). After such a treatment the pattern of expression of the ALK-R1275Q receptor appeared similar to that of the wild-type receptor that is, characterized by a diminution of the lower band of 220 kDa doublet. Therefore, kinase inhibition restored receptor maturation and cell surface localization as demonstrated by biotinylation experiments (Figure 5c). Note that this effect seems less pronounced than for the ALK-F1174L mutant (see Figures 3b and c), but it is in good agreement with the relative ratio of the two bands of the 220 kDa doublet, which characterized the two mutants.

Discussion

ALK has been identified as a critical player in neuroblastoma development and represents a very attractive therapeutic target in this disease (Chen *et al*., 2008; George *et al*., 2008; Janoueix-Lerosey *et al*., 2008; Mosse *et al*., 2008). In particular, two hot spots of ALK-activating mutations at position F1174 and R1275 were found in neuroblastoma sporadic tumor and cell lines (Mosse *et al*., 2009; Janoueix-Lerosey *et al*., 2010), and appeared to be activating in nature. This study clearly establishes that the constitutive activity, cellular localization and trafficking of the

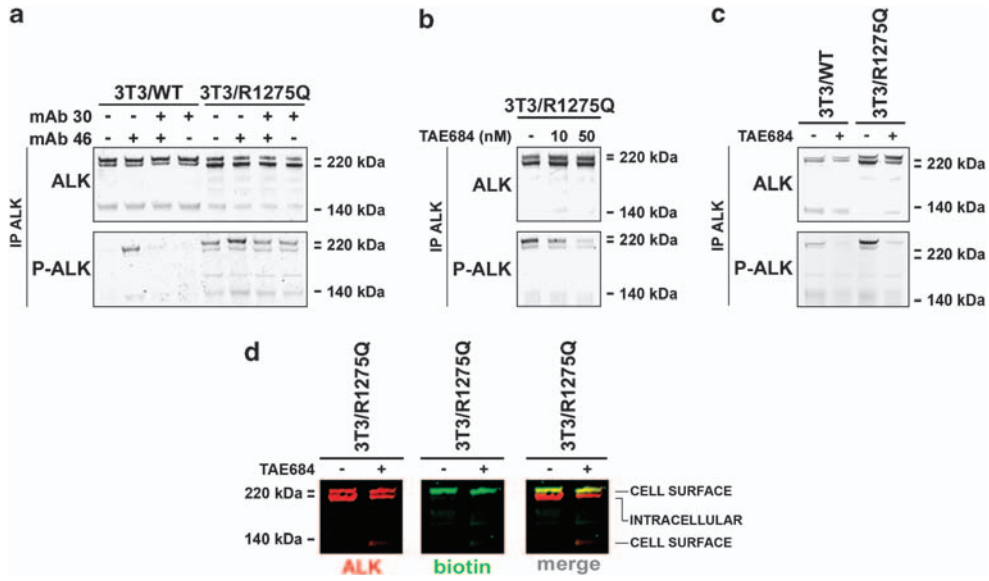

Figure 5 Constitutive activity and cellular localization of R1275Q-mutated ALK. (**a**) 3T3/WT ALK and 3T3/R1275Q cells were untreated (lane 1), treated with 6 nM of agonist mAb46 for 20 min (lane 2), pretreated with 48 nM of mAb30 for 30 min and then with 6 nM of mAb46 for 20 min (lane 3) or treated with 48 nM of antagonist mAb30 for 50 min (lane 4). Cell extracts were prepared by lysing the cells in a RIPA buffer and subjected to immunoprecipitation. Immunoprecipitates were subjected to immunobloting using anti-phosphotyrosine (4G10) or polyclonal anti-ALK (READ) (**b**) 3T3/R1275Q cells were nontreated (lane 1) or treated with 10 nM (lane 2) or with 50 nM (lane 3) of NVP-TAE684 (TAE) for 1 h. ALK immunoprecipitates were subjected to immunoblotting with anti-phosphotyrosine antibody (4G10) and anti-ALK (READ). (**c**) 3T3/WT (lanes 1–2) and 3T3/ R1275Q (lanes 3–4) cells were nontreated (lanes 1–3) or treated with 50 nM of TAE for 24 h (lanes 2–4). ALK immunoprecipitates from cell extracts were analyzed by western blot with anti-ALK antibody (READ) and anti-phosphotyrosine antibody (4G10). (**d**) 3T3/R1275Q cells were incubated (+) or not (−) with 50 nM of TAE for 24 h. They were then subjected to biotinylation procedure as described in Figure 1b. ALK immunoprecipitates were submitted to western blot analysis. Total (in red) and biotinylated ALK (cell surface receptor, in green) were detected as described in Figure 1b.

wild-type and mutated forms of the ALK receptor are different.

We first established that the ALK-WT receptor is only partially addressed to the plasma membrane. In fact the full-length receptor appeared as a doublet of two close ALK molecules of about 220 kDa and the relative ratio of the two bands apparently did not depend of the level of expression of the receptor (different 3T3/WT, IMR-32 or the HEK cell line previously described (Moog-Lutz *et al*., 2005)). We demonstrated that the lower band of the 220 kDa doublet was intracellular and partially processed. In response to endoglycosidase F treatment, molecular masses of both forms of the 220 kDa doublet decreased to about 190 kDa. This indicated that the two bands of the 220 doublet therefore contain the same protein core. Effect of the tunikamycin treatment also deserves a comment. The appearance of the band of about 180 kDa corresponding to the newly synthesized completely non glycosylated receptor was correlated with a strong decrease in the intensity of the lower band of the 220 kDa doublet (Figure 2). Thus, Tunikamycin treatments, as well as biotinylation experiments, strongly support the hypothesis that this band corresponds to the precursor of the mature glycosylated 220 kDa form addressed to the plasma membrane. In NIH3T3 cells expressing ALK-WT, localization at the plasma membrane of the wild-type receptor was detectable but immunofluorescence labeling also showed an intracellular staining. Intracellular localization of ALK in neuroblastoma cells has been already noticed by others (Lamant *et al*., 2000; Osajima-Hakomori *et al*., 2005). Thus, ALK-WT is partially intracellular.

In contrast, the mutated ALK receptors are mainly intracellular and largely retained in the reticulum/Golgi compartments. This intracellular retention appeared more pronounced for the F1174L mutant than for R1275Q mutated receptor. Strikingly, this localization was corroborated by a defect of *N*-linked glycosylation. The present study confirms that the F1174L and R1275Q mutated forms of ALK indeed display a constitutive activation and establishes that kinase inactivation restored maturation and cell-surface localization. Intracellular retention of mutated RTK such as Fms-like tyrosine kinase (Schmidt-Arras *et al*., 2005) and FGFR-3 (Lievens *et al*., 2004) have been already described. Our data therefore support the generality of an inhibitory role of tyrosine phosphorylation in the maturation of RTKs. Note, however, that ALK-WT, in particular, in neuroblastoma cell lines endogenously expressing this receptor, is also largely intracellular. Second and may be more important, to our knowledge, the comparison of the degrees of tyrosine phosphorylation of the intracellular pool and plasma membrane - pool of other mutated RTKs had not been fully documented. As it likely reflects the constitutive activity

of the mutated RTKs, it would be of interest to establish whether, as a general rule, the pool of mutated RTKs addressed to the plasma membrane are much more tyrosine phosphorylated than the intracellular receptors or whether mutated forms of ALK constitute an exception. In fact for the mutated form of the FGFR-3 (Lievens *et al*., 2004), as well as for the mutated variant of the Fms-like tyrosine kinase receptor (Choudhary *et al*., 2009; Buchwald *et al*., 2010), the phosphorylation pattern of the intracellular and plasma membrane pool of these receptors appeared similar to the protein pattern. Thus, from this point of view mutated forms of ALK do not exhibit the same properties than the mutated variants of the two other receptors (and see below).

This constitutive tyrosine phosphorylation of the ALK-mutated receptors deserves several comments. Our antagonist mAb30 had no effect on the basal phosphorylation of the upper band of 220 kDa doublet, suggesting that this constitutive activity did not require the dimerization. It remains, however, to establish whether longer treatment with antagonist mAb could modulate the constitutive activity ALK-mutated receptors located at the membrane. Yet agonist mAbs 46 or 48 increased this basal phosphorylation showing that this constitutive activation can be further increased by an adequate dimerization. An attractive hypothesis is that, because of the transactivation of two molecules of ALK-mutated receptor, additional tyrosine residues were phosphorylated after agonist mAbs treatment. Several hypotheses could be proposed to explain why the minor pool of mutated receptors located at the plasma membrane displays proportionally more tyrosine phosphorylation than the intracellular. One attractive possibility could be that the intracellular pool is permanently much more dephosphorylated than the mature receptors localized at the plasma membrane by cellular proteins tyrosine phosphatases (PTP). In particular, two PTPs have been already shown to interact and negatively regulate ALK tyrosine signaling. The cytosolic PTP SHP1 has been found to interact with the cytosolic oncogenic form NMP-ALK (Honorat *et al*., 2006) and the membrane PTP RPTP beta/zeta with the membrane receptor ALK (Perez-Pinera *et al*., 2007). We are currently investigating whether these PTPs interact with ALK in NIH3T3 cells and differently modulate the phosphorylation of the intracellular and plasma membrane pools of both wild-type and mutated forms of ALK.

In conclusion, the mutated ALK receptors are mainly intracellular, which is characteristic of misfolded protein. They exhibited a clear constitutive basal phosphorylation, which apparently did not require the dimerization of the receptor.

Previous studies of the oncogenic forms of ALK resulting from chromosomal translocations already led to the development of inhibitors of tyrosine kinase activity of ALK (for review (Webb *et al*., 2009)). Thus, the potential use of a drug targeting ALK such as NVP-TAE684 (Galkin *et al*., 2007) in neuroblastoma provides a real hope for future therapeutic treatments for this devastating disease. In cancers involving the EGF receptor as an oncogene, combination of inhibitors of the tyrosine kinase domain and monoclonal antibodies are already routinely used in therapeutic treatments (see for a recent review (Martinelli *et al*., 2009)). Clearly our antagonist mAbs could constitute a therapeutic approach complementary to the inhibitors of TK domain in neuroblastoma in which wild-type ALK is amplified (see for a review (Janoueix-Lerosey *et al*., 2010)). We also established in this study that inhibition of the mutated receptors by TAE led to their translocation to the membrane and we have strong evidences that our mAbs could induce the internalization and down-regulation of both the wild-type and mutated receptors (Mazot and coll in preparation). Thus, even in the absence of amplification, combined to the inhibitors of the TK activity, antibodies may also be used in therapeutic approaches.

Materials and methods

Cell lines, antibodies and reagents

NIH3T3 cells, obtained at the ATCC, were maintained in High-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen, Cergy Pontoise, France) and with 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in atmosphere containing 5% CO_2. Human neuroblastoma cell line CLB-GE has been previously described (Janoueix-Lerosey *et al*., 2008) and the human neuroblastoma cell line IMR32 was a kind gift of Dr P Kogner. They were maintained in RPMI medium supplemented with 10% fetal calf serum.

Constructs in the pcDNA3.1 vectors coding for wild-type ALK and its mutant F1174L and R1275Q were stably expressed in NIH3T3 cells. Stably transfected cells were continuously cultured in the same medium complemented by 400 μg/ml geneticin (Sigma-Aldrich, St Quentin Fallavier, France).

Mouse anti-phospho-ERK1/2 (clone from MAP kinase-YT) was from Sigma-Aldrich. 4G10 anti-phospho-tyrosine and rabbit anti-ERK1/2 were from Millipore (St Quentin en Yvelines, France). Rabbit polyclonal antibodies REAB and mAb 30, 46 and 48 to the extracellular domain of the ALK human receptor have been previously described (Moog-Lutz *et al*., 2005). Zymed rabbit polyclonal anti-ALK was from Invitrogen (Cergy-Pontoise, France), anti-giantin was from Abcam-France (Paris, France) and monoclonal anti-KDEL from Enzo Lifesciences (TEBU, Le Perray en Yvelines, France). The selective ALK inhibitor NPV-TAE684 was synthesized following the formula described in (Galkin *et al*., 2007). Tunikamycin was from Sigma-Aldrich.

Immunoprecipitation and western blotting

Cells washed rapidly with cold phosphate-buffered saline (PBS) (containing 5 mM sodium fluoride and 100 mM sodium orthovanadate) were lyzed in a radioimmune precipitation assay buffer RIPA as described in (Mathivet *et al*., 2007). Lysates were clarified by centrifugation at 21 000 *g* for 10 min at 4 °C. Protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Cell extracts were analyzed by direct immunoblotting or subjected to immunoprecipitation. For immunoprecipitation 1 μg of mouse monoclonal antibodies 48, 30 and 15 (Moog-Lutz *et al*., 2005) were added to 800 μg of protein lysate

and rotated 3 h at 4 °C. A volume of 25 µl of protein G sepharose beads (GE Healthcare Life Sciences, Saclay, France) was then rocked for an additional 2 h at 4 °C. The beads were then washed five times with cold PBS containing 1% Triton X-100. Proteins were then eluted from the beads in 40 µl of SDS/PAGE loading buffer (Invitrogen) by incubating 10 min at 70 °C and subjected to SDS–polyacrylamide gel electrophoresis. After separation in SDS–polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad, Marnes la Coquette, France) for 1 h 30 min at 400 mAmps per gel in 25 mM Tris, pH 8.3, 200 mM glycine and 20% ethanol. The membrane was blocked in PBS, 5% powdered milk and probed with the antibodies at appropriate dilutions for 1 h at room temperature or overnight at 4 °C. After five washing in PBS, 0.1% Tween 20, bound primary antibodies were detected using IRDye 700- or IRDye 800-conjugated second antibodies (Rockland, ME, USA). Binding of the fluorescent antibodies was visualized using the Odyssey Imaging System (LI-COR biosciences, Lincoln, NE, USA).

Immunofluorescence and confocal microscopy

All studies have been performed at the Institut du Fer à Moulin imaging facilities. Cells were fixed for 15 min with 4% paraformaldehyde and washed three times with PBS before being permeabilized using 0.5% PBS and Triton X-100 for 5 min and washed with 0.1 M PBS-Glycine for 15 min. After 1 h of blocking in PBS containing 1.5% bovine serum albumin, cells were incubated in same buffer with, respectively, 1 µg of mAb46/15 and polyclonal anti-giantin or polyclonal anti-ALK (Zymed) and monoclonal anti-KDEL. The cells were then washed five times with PBS before and after incubation with, respectively, anti-mouse IgG Alexa Fluor 488 and anti-rabbit IgG Alexa Fluor 543, or anti-rabbit IgG Alexa Fluor 488 and anti-mouse IgG Alexa fluor 543-conjugated secondary antibodies (Molecular Probes, BP 96, Cergy-Pontoise, France). The cells were then mounted in Vectashield (Vector Laboratories, ABCYS, Paris, France). Confocal laser microscopy was performed using a TCS SP2 confocal microscope (Leica, Gennevilliers, France). Images were assembled using Adobe Photoshop software (DSI, UPMC, Paris, France).

Deglycosylation of ALK with N-*Glycosidase F,* N-*Glycosidase H and* O-*Glycosidase*

For deglycosylation, proteins from immunoprecipitates obtained from NIH3T3 cell lines or neuroblastoma cell line CLB-GE were incubated with *N*-glycosidase F, *N*-Glycosidase H and *O*-Glycosidase (Roche, Mannheim, Germany) following the company instructions. The samples were then used for immunoblotting with anti-ALK antibody.

Cell surface proteins biotinylation

Cell-surface expression level of ALK was examined using the membrane-impermeant biotin EZ-Link Sulfo-NHS-LC-biotin (Pierce). Cells were washed twice in ice-cold PBS, then incubated with biotin for 30 min on ice. After biotinylation, cells were washed with PBS Glycine 0.1 M. After cells lysis in RIPA buffer, proteins extracts were subjected to ALK immunoprecipitation (see above). Cell surface ALK was detected by immunoblotting using a IRDye 800-conjugated streptavidin (Rockland).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)