Unique Substrate Specificity of Anaplastic Lymphoma Kinase (ALK): Development of Phosphoacceptor Peptides for the Assay of ALK Activity†

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ABSTRACT: The anaplastic lymphoma kinase (ALK), whose constitutively active fusion proteins are responsible for 5−10% of non-Hodgkin's lymphomas, shares with the other members of the insulin receptor kinase (IRK) subfamily an activation loop (A-loop) with the triple tyrosine motif Y-x-x-x-Y-Y. However, the amino acid sequence of the ALK A-loop differs significantly from the sequences of both the IRK A-loop and the consensus A-loop for this kinase subfamily. A major difference is the presence of a unique "RAS" triplet between the first and second tyrosines of the ALK A-loop, which in IRK is replaced by "ETD". Here we show that a peptide reproducing the A-loop of ALK is readily phosphorylated by ALK, while a homologous IRK A-loop peptide is not unless its "ETD" triplet is substituted by "RAS". Phosphorylation occurs almost exclusively at the first tyrosine of the Y-x-x-x-Y-Y motif, as judged by Edman analysis of the phosphoradiolabeled product. Consequently, a peptide in which the first tyrosine had been replaced by phenylalanine (FYY) was almost unaffected by ALK. In contrast, a peptide in which the second and third tyrosines had been replaced by phenylalanine (YFF) was phosphorylated more rapidly than the parent peptide (YYY). A number of substitutions in the YFF peptide outlined the importance of Ile and Arg at positions $n - 1$ and $n + 6$ in addition to the central triplet, to ensure efficient phosphorylation by ALK. Such a peculiar substrate specificity allows the specific monitoring of ALK activity in crude extracts of NPM-ALK positive cells, using the YFF peptide, which is only marginally phosphorylated by a number of other tyrosine kinases.

Reversible protein phosphorylation catalyzed by protein kinases and reversed by protein phosphatases is the most common mechanism by which nearly all cellular functions are regulated. Protein kinases constitute a large family of enzymes (>500 encoded by the human genome) (*1*) whose activities are tightly regulated in response to specific stimuli. Consequently, unscheduled and/or deregulated activation of protein kinases, resulting from point mutations, gene rearrangements/amplifications, or metabolic abnormalities, often underlies pathological states, in particular neoplasia (*2*). It is not surprising therefore that protein kinases have recently become an important family of drug targets with several inhibitors of protein kinases already in clinical practice or in advanced clinical trials (*3*). An outstanding example is provided by imatinib (also termed Gleevec, Glivec, or STI-571), an inhibitor of the Abl protein tyrosine kinase (PTK) that is proving extremely successful in the treatment of chronic myeloid leukemia (CML), a clonal hematopoietic stem cell disorder that is characterized by the expression of the constitutively active Bcr/Abl fusion tyrosine kinase (*4*−*9*).

Another example of a PTK that is aberrantly expressed and activated in the form of oncogenic fusion proteins is anaplastic lymphoma kinase (ALK). ALK is a receptor tyrosine kinase normally expressed in specific tissues of the central nervous system with peak expression during embryogenesis (*10*, *11*). Although the function of full length ALK has not been precisely determined, it has been proposed that ALK plays a role in the development and maintenance of the nervous system due to its restricted expression profile. Indeed, activation of an ALK chimera, mimicking ligand activated receptor, was reported to drive differentiation of PC-12 neural cells through stimulation of ERK (*12*). In addition to its expression in the nervous system, ALK is also aberrantly expressed in lymphoid tissues in the form of fusion proteins, resulting from chromosomal translocations involving the *ALK* gene at 2p23 (*13*). ALK fusion proteins are responsible for approximately two-thirds of anaplastic large

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cell lymphoma (ALCL) cases, accounting for 5−10% of all non-Hodgkin's lymphomas (*14*). In addition, ALK fusion proteins have also been detected in about 35% of inflammatory myofibroblastic tumor (IMT) cases (*15*, *16*) and in rare cases of diffuse large B cell lymphomas (DLBCL) (*17*), although a causal role of ALK has not yet been demonstrated in these tumors. To date, eleven ALK fusion proteins have been identified, of which NPM-ALK is the most frequently expressed and studied example (*13*, *18*). NPM-ALK is composed of the N-terminal portion of the ubiquitously expressed nucleolar phosphoprotein nucleophosmin (NPM) and the entire intracytoplasmic region of ALK, containing the catalytic domain. The NPM portion of NPM-ALK contains a homodimerization domain, which is responsible for the formation of NPM-ALK oligomers and consequently the trans-autophosphorylation and constitutive activation of the ALK kinase domain (*19*). NPM-ALK activates anti-apoptotic and mitogenic signaling pathways, such as the PI3-kinase/AKT, JAK/STAT, and PLC-γ pathways (*20*−*22*), resulting in cellular transformation both in vitro and in vivo (*19*, *23*, *24*). Activation of these pathways is mediated via the interaction of SH2 or PTB domains within downstream adapter/signaling molecules with autophosphorylation sites in NPM-ALK. NPM-ALK has been shown to interact with the adapter proteins IRS1 and SHC via the tyrosine residues Y156 and Y567, respectively; however, these interactions are not essential for ALK-induced transformation as no effect was observed upon mutation of these residues to phenylalanine (*13*). In contrast, mutation of the PLCγ docking site Y664 to phenylalanine impaired the mitogenic but not antiapoptotic activity of NPM-ALK. (*22*). PI-3K which mediates the antiapoptotic signaling of NPM-ALK was thought to bind to Y418 of NPM-ALK as this residue is contained in a consensus PI-3K binding motif. However, mutation of Y418 did not impair PI-3K binding to NPM-ALK or its activation, indicating that PI-3K probably interacts indirectly with NPM/ALK possibly via the adapter proteins Gab2, SHC, and/or CrkL (*20*).

Since NPM-ALK and the other variant ALK fusion proteins have a causal role in oncogenesis, they represent a first choice target for the treatment of ALK positive lymphomas. Unlike Abl, which was thoroughly investigated at the protein level prior to the development of specific inhibitors, little is known about the biochemical and catalytic properties of ALK. In particular, the local features determining the substrate specificity of ALK are still unknown. This has hampered the development of sensitive and specific assays for monitoring ALK activity in vitro, which are required for the analysis and development of selective inhibitors. To address this issue we performed an investigation of the substrate site specificity of ALK and developed peptide substrates for optimizing an in vitro assay for measuring ALK activity. The outcome of this study is presented in this report.

MATERIALS AND METHODS

Materials. The monoclonal anti-ALK1 antibody was kindly provided by Dr. K. Pulford (John Radcliffe Hospital, Oxford, U.K.). [γ^{33}P]ATP was purchased from Amersham Pharmacia Biotech (San Francisco, CA) and polyGlu$_4$Tyr from Sigma (Dorset, U.K.). Protease inhibitor cocktail and β-actin antibody were from Calbiochem (Darmstadt, Germany). Genistein, tyrphostin 25, PP2, SU6656, AG490, and AG178 were from Calbiochem; STI571 and staurosporine were kindly provided by Novartis (Basel, Switzerland). All the Edman sequencing reagents and the Sequelon-AA reagent kit (GEN 920033) were from Applied Biosystems (Foster City, CA).

Enzyme Purification. His-tagged recombinant ALK protein containing amino acid residues Leu^{1073}−Ala^{1459} (ALK-HUMAN, Genbank accession code: Q9UM73), including the entire intracytoplasmic domain of ALK except for a few N-terminal residues, was expressed in Sf9 insect cells using the baculovirus expression system, MaxBac 2.0 (Invitrogen), following the manufacturer's instructions. Briefly, cells (2×10^6 cells/mL) were infected with recombinant baculovirus at a multiplicity of infection of 5 and cultured for 72 h at 27 °C. Cells were harvested, washed in ice-cold PBS, and lysed in buffer A (50 mM Tris-HCl pH 8, 20 mM NaCl, protease inhibitors) on ice. Lysate was clarified by centrifugation, and protein was partially purified using a Q-sepharose Fast Flow anion exchange column (Amersham-Pharmacia Biotech), in which proteins were eluted with a linear NaCl gradient (20−200 mM). ALK positive fractions were further purified using a HiTrap-nickel affinity column (Amersham-Pharmacia Biotech) in the presence of buffer B (50 mM Tris-HCl pH 8, 0.5 M NaCl, 50 mM imidazole, 20 mM β-mercaptoethanol, protease inhibitors). Proteins were eluted using a linear imidazole gradient (50−200 mM). Purified protein was aliquoted and stored in 10% glycerol at −80 °C.

The nonreceptor tyrosine kinases c-Fgr, Lyn, Syk, and Csk were purified to near homogeneity from rat spleen as previously described (*25*−*28*). Recombinant human insulin receptor kinase (βIRK) was purchased from Biomol (Butler Pike, PA). Tyrosine kinases were routinely assayed on the random polymer polyGlu$_4$Tyr (molecular mass about 45 kDa) (*26*). One unit of kinase was defined as the amount of enzyme transferring 1 pmol of phosphate/min to 0.1 mg/mL polyGlu$_4$Tyr under standard conditions.

Peptide Synthesis. The peptides derived from the activation loops of ALK and IRK enzymes were synthesized following the solid-phase Fmoc amino acid chemistry (*29*) on an HMP (*p*-hydroxymethyl phenoxymethyl polystyrene) resin (1.1 mmol/g). The chain assembly was performed automatically using a 431 A peptide synthesizer and Fmoc-protected amino acids activated with a mixture of 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), and *N*-ethyldiisopropylamine (DIEA). The side-chain-protected amino acids used were Fmoc-Thr(*tert*-butyl), Fmoc-Ser(*tert*-butyl), Fmoc-Tyr(*tert*-butyl), Fmoc-Tyr[PO(O-benzyl)OH], Fmoc-Cys(trityl), Fmoc-His(trityl), Fmoc-Gln(trityl), Fmoc-Asn(trityl), Fmoc-Lys(*tert*-butyloxycarbonyl), Fmoc-Asp(*tert*-butyl), Fmoc-Glu(*tert*-butyl), and Fmoc-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). Peptide resin cleavage and deprotection was achieved following the procedure of King et al. (*30*). Crude peptides (50−100 mg in 10 mL of water) were purified by a preparative reverse phase HPLC column (prepNova-Pak HR C18, 6 μm, 25 × 10 mm, Waters, Milford, MA) at 12 mL/min. The purity of peptides was >95% as determined by analytical reverse phase HPLC on a 5 μm, C18 Symmetry300 column, 4.6 × 250 mm (Waters).

Peptide Phosphorylation. Peptides were phosphorylated in 30 μL of a medium containing 50 mM Tris/HCl, pH 7.5,

5 mM $MnCl_2$ (5 mM $MgCl_2$ in the case of c-Fgr), 30 μM [γ^{33}P]ATP (specific activity about 1000 cpm/pmol), and the indicated amount of tyrosine kinases. The reactions were terminated at the indicated times by spotting 25 μL of the incubation mixture onto P81 phosphocellulose paper, which was then processed as described elsewhere (*31*). Kinetic constants were determined by GraphPad Prism software fitting the data directly to the Michaelis−Menten equation using nonlinear regression.

ALK Autophosphorylation. ALK autophosphorylation was performed as described for peptide phosphorylation omitting the peptide substrate. Samples were then subjected to SDS−PAGE, and the ^{33}P incorporated in the kinase was analyzed by a Packard Imager.

Solid-Phase Edman Sequencing. ALK and IRK peptides were phosphorylated for 30 min as described above. The reaction mixtures were then diluted to 500 μL with a solution/buffer containing water, acetonitrile (10%), and trifluoroacetic acid (0.1%), and loaded onto a tube containing 50 mg of C18 resin (STRATA from Phenomenex, Torrance, CA). Abundant washings were performed to eliminate exceeding ATP and other mixture components. The phosphorylated peptides were recovered by eluting with 500 μL of an aqueous buffer containing 40% acetonitrile and 0.1% trifluoroacetic acid. Samples were evaporated and resuspended with pure acetonitrile. An aliquot of the resuspended samples, corresponding to 5−10 000 cpm was spotted onto a Sequelon disk (Applied Biosystems, Foster City, CA) and placed on a heated block at 55 °C. Subsequently, coupling of remaining peptides to the membrane was achieved by adding 4 μL of a solution containing *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (10 mg/mL) dissolved in the attachment buffer as described in the Sequelon-AA reagent kit protocol (Applied Biosystems). Sequence analysis was performed on a Procise HT 491 (Applied Biosystems) utilizing a modified Cartridge chemistry cycle to isolate ATZ amino acids according to the manufacturer's instructions. No Flask cycle or HPLC gradient cycles were loaded. At every cycle the removed ATZ amino acid was quantitatively transferred to an external fraction collector connected to the ATZ port, using 90% methanol:10% water as solvent (S1). The collected fractions (600 μL) were counted in a liquid scintillation counter.

Generation of a Stable K299-siALK$_{424}$ Cell Line. The t(2;5)-positive K299 (Karpas 299) ALCL cells were purchased from DSMZ, Berlin, Germany, and cultured at 37 °C with 5% CO_2 atmosphere in RPMI-1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, GibcoBRL, Paisley, U.K.), 100 units/mL penicillin, 100μg/mL streptomycin, and 2 mM L-glutamine (GibcoBRL, Paisley, U.K.).

In order to downregulate ALK activity by inducible-expression of small-interfering RNA (siRNA), K299 cells were stably transfected using Lipofectamine2000 (Invitrogen, Groningen, The Netherlands) with a tetracycline (Tet) repressor expressing vector (pcDNA6)-TR and selected by limiting dilution using blasticidin (10 μg/mL) for 15 days. K299-TR positive cells were subsequently transfected with the pTER-siALK$_{424}$ expression plasmid carrying a doxycycline (Dox)-inducible form of the RNA polymerase III H1 promoter to drive the expression of a hairpin siRNA of 19 bp designed on ALK translocated portion from nucleotide 424 (K299-siALK$_{424}$). Addition of Dox to the culture medium prevented the binding of the Tet repressor to the H1 promoter, and transcription of siALK$_{424}$ was derepressed. The specific siALK$_{424}$ oligonucleotides were synthesized and purified by HPLC (MWG-Biotech, AG) and were as follows: 5′-GATCCCGCTCCGCACCTCGACCATCTTCAAGAGAGATGGTCGAGGTGCGGAGCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGCTCCGCACCTCGACCATCTCTCTTGAAGATGGTCGAGGTGCGGAGCGG-3′. The oligonucleotides (100 pmol of each) were phosphorylated using T4 polynucleotide kinase in a total volume of 50 μL for 30 min, and to anneal them, the mixture was incubated at 95 °C for 5 min and was cooled slowly. One microliter of this mixture was ligated into pTER vector that had been digested with *Bgl*II and *Hin*dIII and treated with calf intestinal phosphatase.

Preparation of K299, SUP-M2 and SUDHL-1, and U937 Cell Lysates. The human anaplastic large cell lymphoma derived t(2:5)-positive, NPM-ALK-positive cell lines, Karpas 299, SUP-M2, and SUDHL-1, and the human histiocytic lymphoma U937 cell line were cultured in RPMI 1640 media (BioWhittaker Europe, Verviers, Belgium), supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin G, 80 μg/mL gentamycin, and 20 mM HEPES, in a humidified atmosphere at 37 °C and 5% CO_2. After centrifugation, cells (8×10^6) were lysed in 450 μL of lysis buffer (50 mM Hepes, pH 7.5, 0.5% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail) for 1 h on ice. After centrifugation (14000*g* for 30 min), protein concentration was determined in the supernatant using the Bradford assay (Pierce, Rockford, IL).

Western Blot Analysis. Purified ALK fractions were subjected to SDS−PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-ALK1 antibody followed by an anti-mouse HRP-conjugated secondary antibody and developed using an enhanced chemiluminescent detection system (ECL, Amersham Pharmacia Biotech).

RESULTS

ALK Displays a More Selective Substrate Specificity than Other PTKs. A portion of ALK (amino acids 1073−1459), which contains the entire ALK kinase domain (amino acids 1116−1392), was expressed as a His-tagged protein using a baculovirus expression system and purified to apparent homogeneity as determined by SDS−PAGE and Coomassie blue staining (Figure 1). Purified recombinant ALK was catalytically active as demonstrated by its ability to undergo autophosphorylation when incubated with phosphoradiolabeled ATP and Mn^{2+} ions (Figure 1). However, recombinant ALK displayed very low activity toward two commonly used, broad specificity, tyrosine kinase substrates, polyGlu$_4$Tyr (a random polymer of glutamic acid and tyrosine) and angiotensin II, compared to other PTKs tested under comparable conditions (data not shown). Furthermore, ALK displayed negligible activity toward a number of peptides developed as substrates for other PTKs, for example the cdc2, HS1 and mT-derived peptides (*32*), which are used for monitoring the activities of Src kinases, Syk and CSK, respectively (data not shown). While this poor activity of ALK could simply

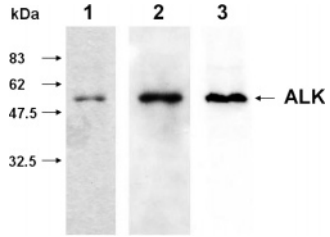

FIGURE 1: Characterization of purified ALK fraction. Sixty nanograms of purified ALK fraction was subjected to SDS−PAGE and either Coomassie blue stained (lane 1) or immunostained with anti-ALK1 antibody (lane 2). Lane 3 shows the autoradiograph of ALK (60 ng) autophosphorylated in the presence of [γ^{33}P]ATP as detailed in Materials and Methods. The molecular mass markers are indicated on the left.

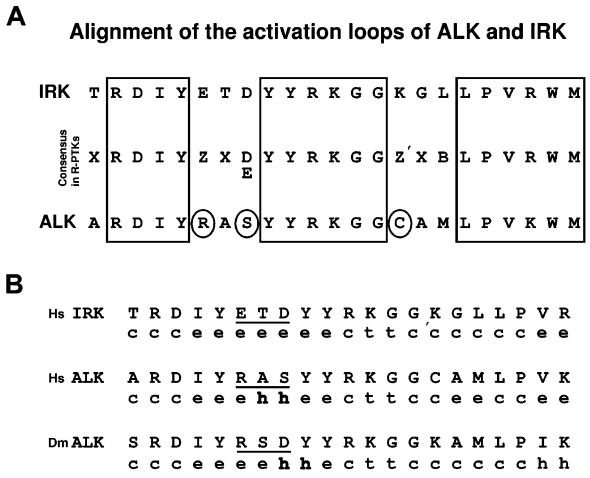

FIGURE 2: Alignment and secondary structure prediction of IRK and ALK A-loops. (A) The consensus was drawn by alignment of receptor tyrosine kinases with Y-x-x-x-Y-Y motif in their A-loops (http://pkr.sdsc.edu/html/index.shtml), namely, Ax1, Ark, c-Eyk, Brt/Sky, IRK, IRR, IGF1R, DILR, LTK, ALK, c-ros, sevenless, Trk, TrkB, TrkC, TorRTK, Ror1, Ror2, Dror, DDR, TKT, MET, and KLG. X, any residue; Z, any residue except arginine; Z′, any residue but never Cys; B, generally hydrophobic. The residues unique to ALK are circled. (B) Secondary structure prediction of human (Hs) IRK and human (Hs) and drosophila (Dm) ALK. The prediction was performed using the SOPMA secondary structure prediction method. t, beta turn; h, helix; c, random coil; e, extend strand.

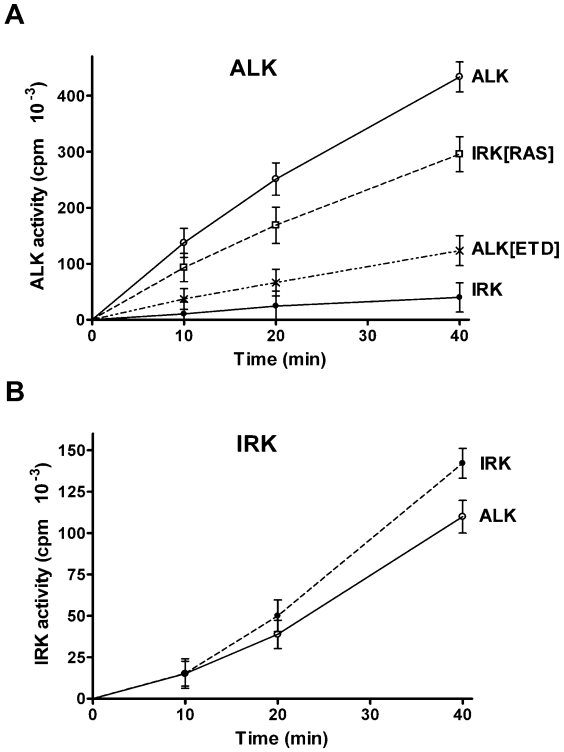

FIGURE 3: Time courses of peptide phosphorylation by ALK and IRK tyrosine kinases. The activity of 60 ng of ALK (A) and 200 ng of IRK (B) tyrosine kinases was tested toward 200 μM ALK (ARDIYRASYYRKGGCAMLPVK) and IRK (TRDIYETDYYRKGGKGLLPVR) peptides as detailed in Materials and Methods. The IRK[RAS] peptide is a derivative of the IRK peptide in which the "ETD" triplet between the first and the second tyrosine has been replaced by the homologous triplet present in the ALK peptide ("RAS"). The ALK[ETD] peptide is a derivative of ALK peptide in which the "RAS" triplet has been replaced by the homologous triplet present in the IRK peptide ("ETD"). Results are mean of four different experiments with SD indicated by vertical bars.

reflect an intrinsic low catalytic efficiency of this kinase, an alternative explanation is that the substrate specificity of ALK is unique compared to other PTKs, and is based on determinants lacking in the peptides tested so far. Evidence supporting the latter possibility comes from sequence analysis of the ALK activation loop (A-loop), which like most PTKs is subject to intermolecular (trans) autophosphorylation, an event that correlates with gain of activity. ALK belongs to a subgroup of receptor tyrosine kinases (R-PTKs), which possess 3 tyrosine residues in their A-loop, conforming to the motif, Y-x-x-x-Y-Y. Alignment of the A-loops of R-PTKs displaying this motif reveals that the ALK A-loop differs significantly from the consensus A-loop sequence for this group of PTKs, which is based on the insulin receptor kinase (IRK) (Figure 2A). In particular the triplet separating the first and second tyrosines contains acidic amino acid residues in IRK ("ETD"), whereas in ALK basic or neutral amino acids are present ("RAS"). Furthermore, the presence of a cysteine residue at position +10 is unique to ALK. These differences in the A-loop sequences of ALK and IRK suggest that the phosphoacceptor substrate specificity of ALK might significantly differ from that of the IRK and other R-PTKs belonging to the Y-x-x-x-Y-Y subgroup, a situation that could be eventually exploited to develop efficient and relatively selective ALK phosphoacceptor peptide substrates.

To check this possibility, two 21-mer peptides encompassing the A-loops of ALK or IRK were synthesized and assayed for their susceptibility to phosphorylation. As shown by the time courses of phosphorylation, ALK readily phosphorylates the peptide which reproduces its own A-loop (amino acids 1274−1294) while it displays very low activity toward the IRK A-loop peptide (Figure 3A). In contrast, IRK kinase is more promiscuous than ALK as it phosphorylates both peptides with similar rates, although it shows a slight preference for its own A-loop peptide (Figure 3B). Note that if the "ETD" triplet in the IRK A-loop peptide is replaced by the "RAS" motif found at the homologous position in the ALK A-loop, phosphorylation of the IRK peptide by ALK becomes comparable to that of the ALK A-loop peptide (Figure 3A). Conversely, as also shown in Figure 3A, the replacement of the "RAS" triplet in the ALK A-loop peptide with the "ETD" triplet dramatically impaired the phosphorylation of ALK peptide by ALK. These data highlight the crucial relevance of RAS triplet in determining ALK selectivity.

ALK Selectively Phosphorylates the First Tyrosine in Its A-Loop Peptide. To analyze in greater detail the differences between ALK and IRK as far as their substrate specificity is concerned, the ALK peptide was phosphorylated by either ALK or IRK and subjected to automated Edman degradation in order to identify which tyrosine(s) were phosphorylated in the two cases. It is already known from experiments with either full length IRK or peptides reproducing its A-loop that the first tyrosine undergoing autocatalytic phosphorylation by the IRK is the second one (Y-1162) followed by the third (Y-1163) and finally by the first (Y-1158) (*33*−*35*). In the case of the ALK A-loop peptide, the first and second tyrosines of the Y-x-x-x-Y-Y motif were phosphorylated to a similar extent by IRK, while the third tyrosine was substantially less phosphorylated (Figure 4A). The outcome with ALK was quite different, with the first tyrosine alone accounting for >90% of the total phosphate incorporated into this peptide (Figure 4B). This result indicates that the isolated first tyrosine in the A-loop is by far preferred by ALK over the YY doublet downstream suggesting an unusual substrate site specificity of ALK, which may have functional consequences regarding its pathogenic potential. This preference for the first Y does not appear to be restricted to the ALK A-loop peptide as the modest phosphorylation of the IRK A-loop peptide by ALK is also almost entirely accounted for by the first tyrosine (Figure 4C). As expected from previous studies (*33*), the second tyrosine in the IRK A-loop peptide was confirmed as the main target of IRK (Figure 4D). A similar phosphorylation pattern was observed with the RET A-loop peptide (SRDV**Y**EEDS**Y**VKRSQGRIPVK), where the first tyrosine was again preferentially phosphorylated by ALK, whereas the second tyrosine was the preferred target of the parent kinase RET (data not shown).

The special relevance of the first tyrosine as compared to the second and third tyrosines was also highlighted by experiments with substituted ALK A-loop peptides in which either Y-1278 or the doublet Y-1282/Y-1283 was replaced by phenylalanine (F) to give the peptides FYY and YFF. As shown in Figure 5, the former substitution caused a dramatic drop in phosphorylation rate, whereas the double substitution was actually beneficial, giving rise to a peptide (YFF), whose phosphorylation is almost 2-fold higher than that of the parent peptide (YYY). In contrast, the YFF peptide is phosphorylated by IRK less readily than the YYY peptide (data not shown). A possible interpretation for the favorable effect of the Y-1282/Y-1283 substitution on ALK catalyzed phosphorylation could be that occupancy of the catalytic site by these tyrosines, which are inefficiently phosphorylated (Figure 4B), might compete against Y-1278 binding and subsequent phosphorylation. This hypothesis is corroborated by the kinetic constants of the YYY vs YFF peptide (Table 1). The superiority of the YFF peptide is accounted for by its V_{max}, which is 3-fold higher than that for the YYY peptide. However, the YFF peptide possesses a K_m that is approximately 4-fold higher than the K_m for the YYY peptide. As binding affinity is inversely proportional to the K_m, this data indicates that the YYY peptide has a greater affinity for ALK compared with the YFF peptide, likely reflecting interactions of the second and third tyrosines with the catalytic site.

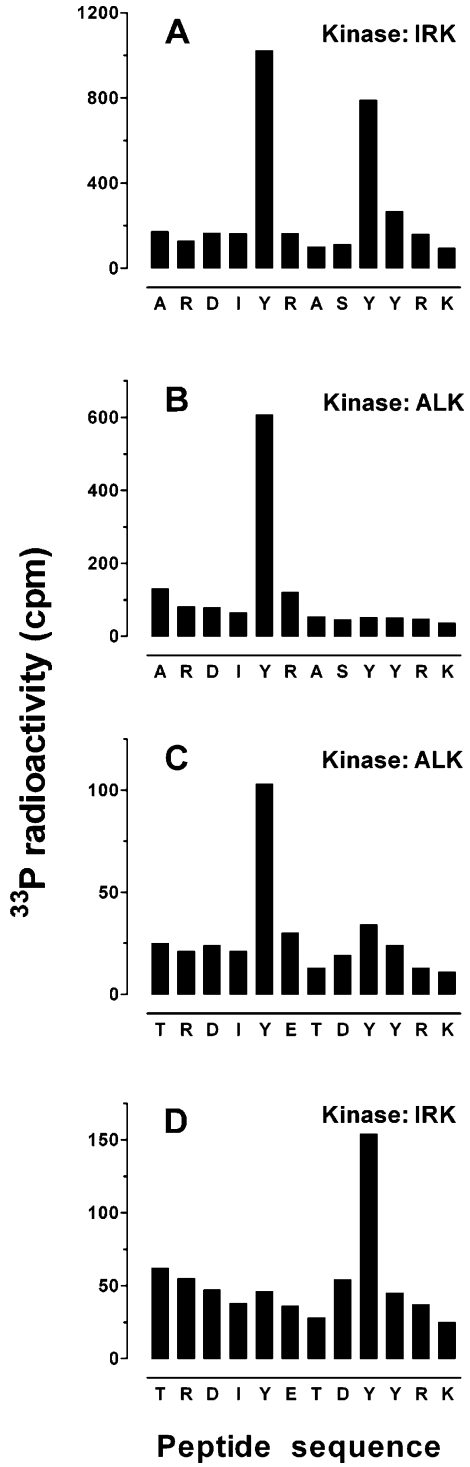

FIGURE 4: Edman sequence analysis of ALK and IRK ^{33}P-peptides phosphorylated by IRK and ALK tyrosine kinases. (A, B) ALK peptide was ^{33}P-phosphorylated for 30 min by either IRK (A) or ALK kinase (B). (C, D) IRK peptide was ^{33}P-phosphorylated for 30 min by either ALK (C) or IRK kinase (D). The radiolabeled peptides were then subjected to solid-phase Edman sequencing as detailed in Materials and Methods. The ^{33}P radioactivity measured at every cycle was plotted versus the primary sequence of the peptide substrate. The figure is representative of three separate experiments.

Identification of Key Residues within the ALK A-Loop Peptide that Define Substrate Specificity. In an attempt to shed light on the local structural features rendering Y-1278 an especially good phosphoacceptor target for ALK, the YFF peptide (number 1 in Table 2) was subjected to a number of

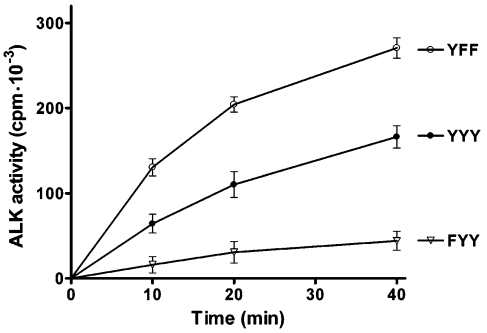

FIGURE 5: Time courses of the phosphorylation of ALK peptide and its derivatives by ALK kinase. Enzyme and peptide concentrations were 70 nM and 200 μM, respectively. Similar data were obtained by replacing the Leu1073−Ala1459 ALK fragment used in this study with another recombinant ALK protein encompassing residues Leu1073−Lys1410. Results are mean of four different experiments with SD indicated by vertical bars.

Table 1: Kinetic Constants for Peptide Phosphorylation by ALK Tyrosine Kinase

peptide	V_{max} (pmol/min)	K_m (μM)
YYY	20.3	89
YFF	62.3	340

Table 2: Phosphorylation Rates of ALK(YFF) Peptide Derivatives by ALK Tyrosine Kinase[a]

Derivative	Sequence	Phosphorylation rate
	1278	
1	ARDI**Y**RASFFRKGGCAMLPVK	**100.0**
2	AADI**Y**RASFFRKGGCAMLPVK	**61.7**
3	ARAI**Y**RASFFRKGGCAMLPVK	**64.7**
4	ARRI**Y**RASFFRKGGCAMLPVK	**55.8**
5	ARDA**Y**RASFFRKGGCAMLPVK	**11.7**
6	ARDI**Y**AASFFRKGGCAMLPVK	**105.8**
7	ARDI**Y**EASFFRKGGCAMLPVK	**56.5**
8	ARDI**Y**RAAFFRKGGCAMLPVK	**103.0**
9	ARDI**Y**RASFFAKGGCAMLPVK	**3.0**
10	ARDI**Y**RASFFRAGGCAMLPVK	**27.9**
11	ARDI**Y**RASFFAAGGCAMLPVK	**2.4**

[a] Synthetic peptides (400 μM) were phosphorylated by 10 units of ALK for 15 min as detailed in Materials and Methods. Phosphorylation rates are expressed relative to peptide YFF. The phosphoacceptor tyrosine residue is in boldface type, and substitutions relative to peptide YFF are underlined.

substitutions, the consequences of which were monitored with respect to phosphorylation rate. From the data presented in Table 2 it appears that the hydrophobic isoleucine (I) at position $n - 1$ relative to Y-1278, and the two basic residues (R and K) at positions $n + 6$ and $n + 7$, with particular reference to K at $n + 6$, are important determinants in defining substrate efficiency, as their replacement by alanine (A) almost completely abolished peptide phosphorylation (compare peptides 1, 5, and 9). In contrast, the individual replacements of R at $n - 3$, D at $n - 2$, R at $n + 1$, and S at $n + 3$ with A had a less marked or no effect on phosphorylation rates. However, the key role of the unique

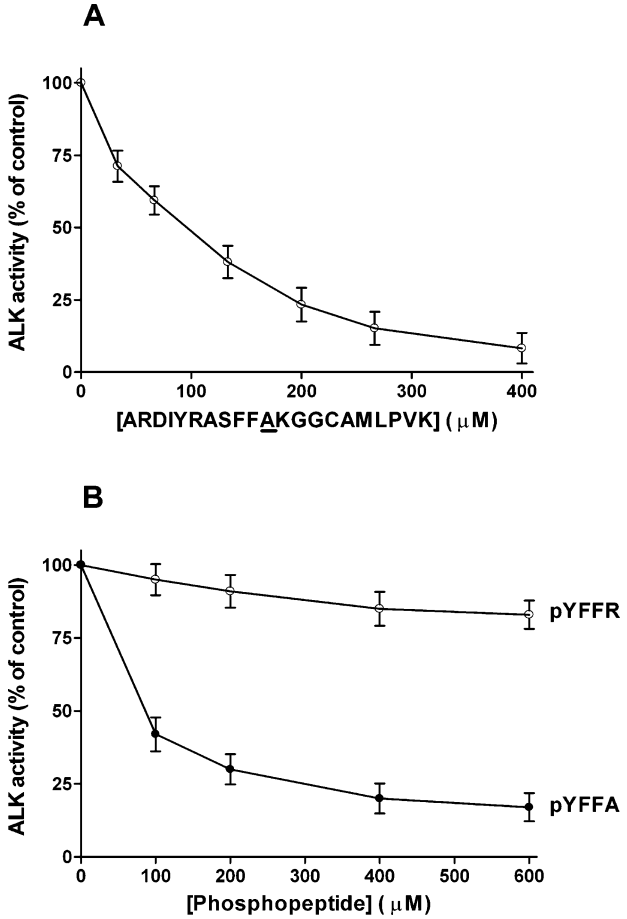

FIGURE 6: Role of Arg($n + 6$) in the kinetics of YFF peptide phosphorylation.The activity of 60 ng of ALK tyrosine kinase was tested toward 200 μM YFF peptide in the presence of increasing concentrations of either the YFF derivative, ARDI**Y**RASFFAKGGCAMLPVK, containing Arg($n + 6$) replaced by Ala (A) or the phosphopeptides (B) ARDI**pY**RASFFRKGGCAMLPVK (open circles) or ARDI**pY**RASFFAKGGCAMLPVK (solid circles).

"RAS" triplet immediately following Y-1278 of ALK is corroborated by two findings: first, the replacement of R at $n + 1$ with glutamic acid (E), the corresponding amino acid in the IRK A-loop peptide, promotes a 50% decrease in phosphorylation rate (compare peptides1 and 7, Table 2); second, as outlined above, the replacement of the "RAS" triplet with the "ETD" motif found in IRK promotes a dramatic drop in phosphorylation rate (Figure 3A).

The crucial role of the R at position $n + 6$, whose replacement with A totally abrogates phosphorylation (peptide 9, Table 2), was especially intriguing considering that this residue is relatively remote from the target tyrosine and it is conserved in most receptor protein tyrosine kinases. Despite the fact that peptide 9 is not phosphorylated by ALK, it still appears to bind efficiently to ALK as it is able to inhibit in a dose-dependent manner the phosphorylation of the YFF peptide (Figure 6A), suggesting that R($n + 6$) is not required for substrate recognition. It is possible that R($n + 6$) accelerates the catalytic reaction by facilitating the release of the phosphorylated product, which is the rate-limiting step of the protein kinase reaction. This may occur through a conformational change driven by the formation of a salt bridge with the phosphorylated side chain, as observed with the phosphorylated products of other kinases (*36*). To test the plausibility of this hypothesis, two phos-

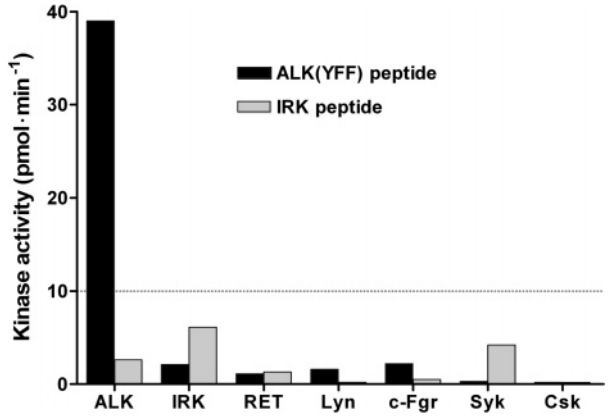

FIGURE 7: Activity of different tyrosine kinases on ALK(YFF) and IRK peptides. 400 μM peptides were phosphorylated by 10 units of the indicated tyrosine kinases as detailed in Materials and Methods. The dotted line denotes the activity of the different tyrosine kinases on polyGlu$_4$Tyr. Data are the mean of four separate experiments. SEM values were always less than 16%.

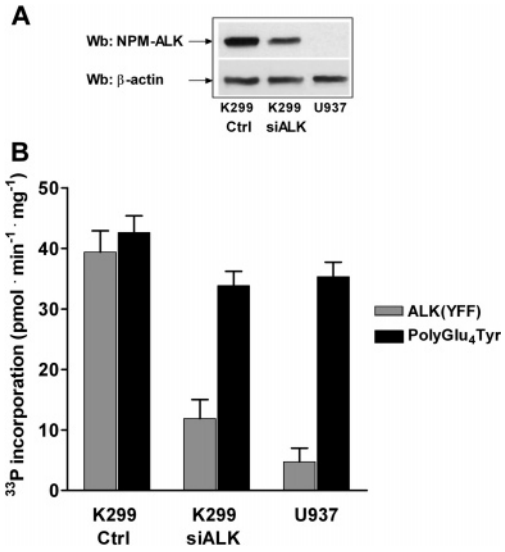

FIGURE 8: Detection of ALK activity in NPM-ALK positive or negative cell lysates. The lysates of human anaplastic large cell lymphoma cells, K299 (NPM-ALK positive), control (Ctrl) or containing silenced ALK RNA (siRNA), and U937 (NPM-ALK negative) were prepared as detailed in Materials and Methods. The tyrosine kinase activity of cellular lysates (6 μg total protein) was monitored on either 200 μM YFF peptide (gray bars) or 1 mg/mL polyGlu$_4$Tyr (black bars). Results are mean of four different experiments with SD indicated by vertical bars. The inset shows the Western blot analysis of control and siALK K299, and U937 cell lysates (30 μg). Blots immunostained with anti-ALK1 antibody were reprobed with β-actin antibody for loading control.

phopeptides were synthesized, corresponding to the phosphorylated products of either the YFF peptide (peptide 1, Table 2) or its derivative with R(n + 6) replaced by A (peptide 9, Table 2). These peptides were tested as inhibitors of ALK. The results shown in Figure 6B clearly demonstrate that the phosphopeptide lacking R(n + 6), but not the control YFF phosphopeptide with R(n + 6), readily inhibits the kinase reaction, consistent with our hypothesis. This shows that only the YFF phosphopeptide, but not its substituted derivative lacking R(n + 6), adopts a low affinity conformation expected for a product able to be promptly released from the active site of ALK.

Use of the YFF Peptide for the Specific Measurement of ALK Activity in Crude Cell Extracts. The excellent performance of the YFF A-loop peptide may be exploited to set up a sensitive assay for measuring ALK activity. The presence of 5 naturally occurring basic residues in this peptide allows the use of anionic phosphocellulose paper for the separation of the phosphoradiolabeled peptide from excess radioactive ATP in the reaction mixture. In order to determine how selective such an assay would be for monitoring ALK activity, the YFF peptide was tested against a panel of PTKs using equal units of enzyme. Enzyme units were defined toward polyGlu$_4$Tyr, a broad specificity substrate used for testing all tyrosine kinases. The data presented in Figure 7 show that under our test conditions (200 μM peptide substrate and 30 μM ATP) the YFF peptide was by far preferred over polyGlu$_4$Tyr by ALK, but not by other PTKs. This selectivity was not observed with the IRK A-loop peptide.

Consequently, the YFF peptide may serve as a tool to detect and quantify the activity of ALK in crude cell extracts containing other PTKs. To check the feasibility of this approach, lysates from NPM-ALK positive (K299) and negative (U937) cells were tested for their ability to phosphorylate either polyGlu$_4$Tyr or the YFF peptide. No significant difference in tyrosine kinase activity was detected between the two cell lines using the broad specificity polyGlu$_4$Tyr substrate, while the activity detected using the YFF peptide was more than 7-fold higher in the K299 cell lysates compared with U937 (Figure 8). The residual activity toward the YFF peptide in U937 cell lysates might be due to ubiquitous protein tyrosine kinases (notably those of the Src family), which display detectable albeit modest activity toward this peptide (see Figure 7). To corroborate the conclusion that the phosphorylation of the YFF peptide observed with K299 cell lysates is mainly due to the high expression and activity of NPM-ALK rather than other tyrosine kinases present in K299 but not in U937 cells, the experiment was repeated with two other NPM-ALK positive cell lines, SUP-M2 and SUDHL-1. Again the activity monitored with the YFF peptide but not with polyGlu$_4$Tyr was 3- to 5-fold higher than in control U937 cells not expressing ALK (data not shown**).** Moreover, a number of inhibitors of a wide spectrum of tyrosine kinases, namely, genistein, tyrphostin 25, PP2, SU6656, AG490, AG178, and STI571, all of which are ineffective on ALK, do not affect the phosphorylation of the YFF peptide by K299 cell lysates (data not shown). In contrast, staurosporine, a broad specificity kinase inhibitor, which we have observed to be very efficient on ALK, as well as the inhibitory phosphopeptide shown above (pYFFA) were both able to inhibit YFF phosphorylation by K299 cell lysates (not shown). These results leave little doubt that the activity monitored with the YFF peptide in K299 cell lysates is mainly due to ALK activity. The formal demonstration that this is really the case was provided by silencing ALK RNA in K299 cells: as shown in Figure 8, this treatment reduced by about the same proportion the expression of ALK protein and the activity monitored with the ALK A-loop derived YFF peptide.

DISCUSSION

There are around 550 protein kinases encoded by the human genome (*1*) which are collectively committed to the phosphorylation of several thousand proteins, based on the

estimate that about one-third of the whole eukaryotic proteome is phosphorylated. This highlights the incredible specificity of each protein kinase, permitting the phosphorylation of a small minority of potential targets, while avoiding misphosphorylations that could have deleterious consequences. Among the several factors contributing to the selectivity of protein kinases, the ability to recognize consensus sequences specified by local structural determinants within the substrate plays a major role, especially in the case of Ser/Thr protein kinases (*37*). Hence, fairly reliable predictions can be made about the sites potentially phosphorylated by a given kinase (see, e.g., phospho.elm.eu.org and scansite.mit.edu). Furthermore, highly specific peptide substrates have been developed for monitoring the activity of individual kinases or classes of kinases (*32*).

In the case of tyrosine specific kinases, however, substrate specificity appears to be less pronounced, with targeting being predominantly dictated by docking modules and interacting domains outside the catalytic core (*37*). Consequently, consensus sequences strictly recognized by individual PTKs have been reported in only a few cases, and peptide substrates developed for a particular PTK are often phosphorylated by other PTKs (*38*). Especially noteworthy in this respect are the random polymers of tyrosine and glutamic acid, and the peptide hormone angiotensin II, both of which are widely used as broad specificity phosphoacceptor substrates for a variety of receptor and nonreceptor PTKs.

Despite these premises, it has clearly been shown that subtle differences do exist in the recognition of local specificity determinants for individual PTKs (*39*, *40*) and that alteration of these properties can lead to aberrant phosphorylation of protein targets and potentially disease (*39*). Therefore, the definition of the local structural determinants recognized by an individual PTK may provide useful information especially in the case of oncogenic kinases like ALK, whose constitutively active fusion proteins are responsible for 5−10% of non-Hodgkin's lymphomas. In addition, such an investigation should allow the development of a specific peptide substrate for the sensitive monitoring of ALK activity and for the assay of potential ALK inhibitors.

ALK belongs to a subfamily of R-PTKs, which includes IRK and is characterized by three phosphorylatable tyrosines in the A-loop, displaying the motif Y-x-x-x-Y-Y. Our data, obtained with a set of synthetic peptide substrates derived from the ALK and IRK A-loops, show that the specificity of ALK is unique in several respects. First, sequence alignment of the A-loops of PTKs possessing the Y-x-x-x-Y-Y motif reveals that the ALK A-loop differs significantly from the consensus A-loop for this group of PTKs. Second, at variance with IRK and presumably most Y-x-x-x-Y-Y PTKs, ALK does not appreciably phosphorylate the second and third tyrosines of the A-loop, but almost exclusively targets the first isolated tyrosine (Y1278). Third, phosphorylation of this tyrosine by ALK is further enhanced if the second and third tyrosines are replaced by phenylalanines to give the YFF peptide. Fourth, phosphorylation of Y1278 in this peptide is drastically reduced by a number of local alterations, notably the replacement of R1284, K1285, and I1277 by alanine and to a lesser extent by the replacement of R1279 by glutamic acid, i.e., the amino acid found at the homologous position in the IRK.

Most likely, R1284 plays a crucial role by interacting with the phosphate of Y1278 and thus inducing a conformational change essential for the release of the phosphorylated product. Both the nonphosphorylated and phosphorylated forms of the substituted peptide with R1284 replaced by alanine, in fact, behave as powerful inhibitors of the phosphotransferase reaction, denoting high affinity for the catalytic site, which is entirely lacking in the case of the phosphorylated product of the YFF peptide. The detrimental effect of replacing R1279 with glutamic acid on the other hand may partially account for the poor phosphorylation of the IRK peptide by ALK. It is likely, however, that differences in the overall conformation of the ALK vs IRK derived peptides are mainly responsible for their different susceptibility to phosphorylation by ALK. This hypothesis is corroborated by secondary structure predictions suggesting that, while in the IRK peptide the three tyrosines belong to an uninterrupted strand, in the ALK peptide the first tyrosine is separated from the other two by a short helical element (Figure 2B) expected to impose a bend in the middle of the peptide. Interestingly, in the *Drosophila* homologue of ALK, which also activates the ERK/MAP kinase pathway (*41*) upon stimulation by its ligand Jeb (*42*) and in which the "RAS" motif is replaced by an "RSD" triplet, the predicted helical bend is still conserved (see Figure 2), suggesting that a bend between the first and second tyrosines is instrumental to ALK functionality. Note that in the crystal structure of inactive IRK (*35*) the activation loop does not display any helical feature, whereas the helical bend between Y1278 and Y1282 becomes evident in the activation loop of ALK modeled on the IRK structure (not shown). Y1278 points outward accessible to the catalytic site of another ALK molecule and, once phosphorylated, to the SH2 domain of another molecule. Consistent with the different conformation of the ALK vs IRK peptide is also the observation that IRK, which preferentially phosphorylates the second tyrosine in its own A-loop peptide, phosphorylates both the first and second tyrosines to equivalent degrees in the ALK peptide (compare Figure 4A and Figure 4D).

The relevance of the individual sequence determinants described here for the synthetic ALK A-loop peptide, to the phosphorylation of the A-loop in native ALK protein, remains to be established. However, a number of recent observations with native ALK proteins support our finding that the first tyrosine in the ALK A-loop, Y1278, is preferentially phosphorylated, suggesting that our results with the synthetic A-loop peptides may indeed reflect a physiological situation. First, mutation of tyrosine residues in the A-loop of full length NPM-ALK, expressed in murine pro-B BaF3 cells, revealed that the double mutant, Y342F/Y343F (equivalent to the YFF synthetic peptide in Figure 5), is tyrosine phosphorylated at a level comparable to wild-type NPM-ALK. In contrast, the single mutant, Y338F (equivalent to the FYY synthetic peptide in Figure 5), shows no detectable tyrosine phosphorylation (C. Gambacorti-Passerini, personal communication). Second, a recent phosphoproteomic analysis of Karpas 299 cells (*43*) has identified four phosphopeptides variably encompassing the A-loop of ALK: none of these included phosphorylated Y1283, and only one included phosphorylated Y1282, while the first tyrosine, Y1278, was phosphorylated in all four peptides,

consistent with the phosphorylation pattern of the synthetic A-loop peptide described here (Figure 4).

The unique specificity of ALK is reflected by the fact that the YFF peptide substrate, which is readily phosphorylated by ALK, is instead a relatively poor peptide substrate for a panel of 7 different PTKs, tested under conditions where their activities were normalized toward the broad specificity substrate polyGlu$_4$Tyr. This finding, in conjunction with the testability of this peptide by the classical phosphocellulose paper procedure, and its successful application to the monitoring of ALK activity in crude cell extracts, provides the rationale for setting up a sensitive, easy, and fairly specific assay of ALK activity.

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