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Review

Tyrosine protein kinase assays

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Abstract

Protein kinases form a large family of enzymes that play a major role in a number of live processes. The study of their action is important for the understanding of the transformation mechanisms and of the normal and pathological growth events. The quality of an enzyme assay is often the key point of an enzymatic study. It must be flexible and compatible with various experimental conditions, such as those for the purification process, the screening of inhibitors and the substrate specificity studies. As will be shown in the present review, two categories of substrates, peptidic and proteic, should be distinguished. The use of peptide substrates facilitates the determination of the recognition requirements of the enzyme and of the kinetic effects of even minute variations in their sequence. These linear peptide structures are assumed to mimic a complex interaction between the enzyme and a proteic substrate in which distant amino acids in the sequence are vicinal in the folded substrate. Less amenable to a systematic study, but probably more adequate to investigate the natural substrate of a given kinase, are the proteic substrates. Obviously the tools to measure protein kinase activities are not the same in these two cases. The main difficulty in assaying protein kinases is the use of labelled γ-ATP, mostly at large excess concentration, since the final product of the reaction has to be separated from the non-reacted labelled ATP. In the case of peptide substrates, the difficulty is to separate them from ATP basing on differences of molecular mass. Despite the efforts of many investigators to rely upon differences in solubility, in charges or in "affinity", this separation, which is crucial for the assay, is still an unsolved experimental problem. Chromatographic, as well as electrophoretic assays appeared relatively late in this domain, and more work in assessing new methodologies might bring new breakthroughs in the next few years. Specific, simple and reliable kinase assays are still a major challenge. Their improvement will help to conduct specificity studies, to elucidate complex growth mechanisms in which they are involved and to discover more selective potent inhibitors.

Keywords: Reviews; Tyrosine protein kinase; Enzymes

Contents

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1. Background: Why assaying tyrosine protein kinases?

1.1. TPK in live processes: multiplicity

Tyrosine protein kinases form a family of enzymes implicated in second signalling pathways and growth functions (for review, see Ref. [1]), intracellular signalling [2], cell cycle [3], immune response [4], to name only a few. Their functions are often related to growth processes and they are regulated by many different factors: cAMP, cGMP, single-strand DNA, phospholipids, growth factors, interferon, calcium, etc. Tyrosine protein kinase activity is also catalysed by numerous oncogene products implicated in various types of cancer [5].

On the basis of their subcellular distribution, protein kinases belong to three different types: transmembrane (receptors) [6], membrane-associated [7] and cytosolic proteins [7]. It appears that most receptors of the growing factors do start their respective signalling pathways by a protein-kinase-mediated phosphorylation of an intracellular target followed by a physical association between the enzyme and its phosphorylated substrate. Alternatively, phosphorylation would activate or deactivate the target protein (see $p42^{mapk}$ regulation by $p56^{lck}$-mediated phosphorylation [8]). Some other receptors recrute, as a first step, an otherwise cytosol-associated protein kinase (e.g., $p56^{lck}$). Due to their involvement in so diverse biochemical processes, protein kinases have received a great deal of interest during the last decades (see, for review, Refs. [9–11]) and appear to be a crucial target for signal-transduction therapy, particularly, but not only, in cancer [7]. There are literally hundreds of protein kinases [12]. On the basis of their sequences, it has been possible to construct a family tree of these enzymes [13,14]. This extraordinary diversity is also due to the presence of such enzymes in almost any living organism: from nematodes [15], planarians [16] and sponges [17] to mammals.

1.2. TPK as a molecular tool: difficulties

One of the main goals of studying protein kinases is finding specific inhibitors. However, because of the large number of existing protein kinases, it is necessary to check the specificity of a given inhibitor on a series of protein kinases and/or to compare the different protein kinase specificities. Studies have been conducted using one or several protein kinases under highly variable conditions using new or already described assays. The origin of the inhibitors in these screenings included microbiological broths (e.g., Ref. [18]), other natural sources [19] and even synthetic families of compounds (see, for example, Refs. [20–22]). Flavonoids [23], and later on tyrphostins [24] seemed to be new classes of promising compounds in anticancer therapy. Finally, other agents were discovered, like PD153035, a picomolar inhibitor of the EGF receptor tyrosine protein kinase, a lead in a new class of potent and efficient anticancer drugs [25].

Because the specific activity of TPKs is often very low (a hundredth of the activity of serine/threonine protein kinases), their purification has been a difficult task. In addition, they are often unstable and poorly

represented in a given biological sample (tissues or cells) and co-exist in several forms in most biological sources (cf., the 14 putative tyrosine protein kinases found in K562 cells using molecular biological techniques [26]). For instance, the purification of the four main cytosolic TPKs from the human promyelocytic cell line HL60 was a long and tedious process which was only partially achieved in our laboratory [27,28]. Comparison of the TPKs isolated from various sources was difficult on the basis of solely biochemical characteristics and finally required a complete amino acid sequence determination. Nevertheless, an impressive number of protein kinases have been sequenced (see list in Ref. [29]).

1.3. Access to TPK activity measurement

TPK activity can be measured either by their substrate phosphorylation capacity (i.e., the ability to transfer a phosphoryl group from ATP (or GTP) onto the hydroxyl residue of an amino acid within a peptidic or proteic sequence), or by their autophosphorylation capacity (i.e., the ability to transfer a phosphoryl group onto an own hydroxyl residue or to a parent kinase, the interphosphorylation capacity of the protein kinases associated to growth factor receptors). Assays of tyrosine protein kinases have been surprisingly rarely based on standard HPLC methodologies. Most of the methods are derived from a solid-phase extraction procedure using phosphocellulose as the solid support. Nevertheless, the studies of a particular feature of TPK (autophosphorylation) and to a certain extent, of the phosphorylation of proteins (as opposed to peptides) are primarily based on electrophoretic methods followed by autoradiography. The extraordinary diversity of the pathways in which protein kinases (particularly tyrosine protein kinases) are involved are reflected at the molecular level by their restricted specificities towards cellular targets [30]. Therefore, knowledge of the amino-acid sequences of their respective protein substrates is important in order to understand the restricted specificity of protein kinases. However, the use of peptidic sequences to study the specificity of protein kinases has to be proven at the in vivo level [31]. Casnellie and Krebs [32] first conducted systematic studies of short peptidic sequence phosphorylation with the aim to find the best fitting and shortest possible substrate which ultimately could be chemically modified into a specific inhibitor [33]. Nevertheless, the requirement of having both a short and potent peptidic structure seems to be difficult to fulfil. Fig. 1 clearly indicates that the K_m is inversely proportional to the length of the putative substrate: the shorter the structure, the higher the K_m. This observation is based on studies reported in the literature and includes various enzymes treated as a whole and, therefore, leads only to a general tendency [11].

1.4. TPK assays: the statement

In fact, assaying TPK can be summarised by a single statement: the key is the separation of the labelled ATP from the phosphorylated substrate; the shortest the peptide substrate, the harder the separation. Indeed, the measurement of TPK activity has two steps: an incubation assay, the composition of which can varies from enzyme to enzyme but also from laboratory to laboratory, and the assay itself which consists of the use of a technique to separate the product of the reaction (i.e., the phosphorylated peptide) from the rest of the components of the incubation medium. A brief description of the available assays is given in Table 1.

2. Assaying tyrosine protein kinase: experimental tricks and non-exhaustive listing

The key point for the characterisation, purification and screening of inhibitors of any enzyme is the availability of a reliable, simple and fast assay which can at the same time handle large series of samples.

2.1. Techniques for measuring TPK

There are basically four ways to obtain evidence of the presence of these enzymes in a given biological preparation (cell and tissue homogenates):

1. Immunological methods, enabled in part by the commercial availability of numerous monoclonal antibodies raised against several TPKs.

Table 1
Tyrosine protein kinase assays

Volume	Typical peptide structure and concentration		Buffer, pH	Cation	Adjuvant(s)	ATP	Stop	Post-assay conditions	Buffers[c]	Refs.
Ion-exchange chromatography/extraction[a]										
50 µl	EDNEYTA, EPQYQPA and derivatives	50 µg	50 m*M* Tris, 7.5	10 m*M* $MgCl_2$	10 µ*M* vanadate, 0.05% Triton	20 µ*M*	50 µl 5% TCA	Hydrolysis, 1 *M* HCl, boiling, ion-exchange chromatography on AG 1, complexation with molybdate, extraction by isobutanol–benzene, counting	(e) 30% acetic acid	[47,48]
Chelation/PEI-cellulose ion exchange chromatography										
50 µl	DRVYVHPF RDRVYVHPF	4 m*M*	150 m*M* HEPES, 8.0	6 m*M* $MgCl_2$	3 m*M* $HS(CH_2)_2OH$, 10% glycerol, 0.01% Triton X-100	200 m*M*	50 µl 27 m*M* acid molybdate	Centrifugation, chromatography on PEI cellulose, elution by centrifugation, counting	(e) 1 *M* LiCl	[50]
DEAE-Sephacel/ferric chelation column										
300 µl	Library	1 mg	50 m*M* Tris, 7.5	10 m*M* $MnCl_2$	1 m*M* DTT, 50 m*M* NaCl	100 µ*M*	Filtration through Microcon-10	Dilution with acetic acid (30%), first chromatography on DEAE-column, lyophylization, second chromatography on IDA beads, sequencing	(e1) elution in 30% acetic acid (e2) elution in NH_4CO_3, pH 8.0	[106]
Reversed-phase HPLC										
25 µl	RRLIEDAEYAARG	2.5 m*M*	12.5 m*M* HEPES, 7.4	10 m*M* $MnCl_2$		50 µ*M*	10 µl 100% TCA	Addition of 1.5 ml NaH_2PO_4, analysis on C_{18} column, collection of 1-ml fractions counting	(e) ATP and Pi eluted in 4.8% NaH_2PO_4, and then gradientwise to 50% acetonitrile	[54]
100 µl	DRVYIHPF and derivatives	3 mg/ml	20 m*M* HEPES, 7.4	5 m*M* $MgCl_2$		10 µ*M*	500 µl 10% TCA + 10 m*M* NaPP	Centrifugation, analysis on phenyl-µBondapak column, (counting on line)	(e) Gradient-wise between 40 m*M* Na phosphate, pH 7.4, 5 m*M* TBAP, acetonitrile (90:10 and 70:30)	[51]
50 µl	EKIGEGTYGVVYK and derivatives	300 µ*M*	50 m*M* Tris, 7.0	50 m*M* $MgCl_2$	2 m*M* DTT, 40 m*M* NaCl	100 µ*M*		SPE on C_{18} Sep-Pak to remove free ATP, evaporation, chromatography on Vydac C_{18}, collected, counting	(e) gradient-wise between water/TFA (0.1%) and acetonitrile–TFA (0.1%)	[53]
On-line liquid chromatography-electrospray mass spectrometry[b]										
500 µl	RRLIEDAXYAARG 19 analogs at the time	2.7 m*M*	62 m*M* Tris, 7.4	12 m*M* $MgCl_2$	1.8 mg/ml BSA	250 µ*M*	Filtration through Microcon-10	Analysis on Vydac C_{18} column, post column splitting, ESMS detection	(e) Gradient-wise between water/TFA (0.1%) and acetonitrile–water–TFA (75:25:0.1%)	[52]
Hydrophilic chromatography										
100 µl	Any peptide	3 mg/ml	20 m*M* HEPES, 7.4	5 m*M* $MgCl_2$, 5 m*M* $MnCl_2$		10 µ*M*	40 m*M* TEAP, pH 2.8–acetonitrile 500 µl (v/v)	Centrifugation, analysis on polyHYDROXYETHYL aspartamide column, counting on line	(e) Gradient-wise between 4 m*M* TEAP–acetonitrile (10:90) and 10 m*M* TEAP, pH 2.8	[39]
Anion-exchange thin-layer chromatography										
100 µl	YIYGSFK	8–2400 µ*M*	30 m*M* MES, 6.8	10 m*M* $MgCl_2$, 0.4 mg/ml BSA		100 µ*M*	150 m*M* phosphoric acid	TLC on Cellulose Mn 300, autoradiography	(t) 15 m*M* phosphoric acid	[102]
Thin-layer electrophoresis										
8 µl	KLIEDNEYTAR and derivatives		20 m*M* PIPES, 7.0	10 m*M* $MgCl_2$		40 m*M*	Cooling	Centrifugation, electrophoresis (100 µm cellulose thin-layer plate), drying, autoradiography	(a) Pyridine–acetic acid–water (1:10:489) pH 3.5, 1000 V	[42]

High-voltage paper electrophoresis										
25 μl	DRVYIHPF and derivatives	2 m*M*	25 m*M* MES, 6.5	10 m*M* $MnCl_2$	10% glycerol	40 μ*M*	Heating, 90°C	Centrifugation, high-voltage paper electrophoresis, drying, autoradiography	(e) Pyridine–acetic acid–water (1:10:189) pH 3.5, 3000 V	[43]
SDS-gel electrophoresis										
100 μl	KIGTAEPDYGALYEGR among others	10 μ*M*	50 m*M* HEPES, 7.5	10 m*M* $MnCl_2$	150 m*M* NaCl, 0.1% Triton X-100, 10% glycerol	10 μ*M*	Equal volume of 2×SDS buffer	Electrophoresis on 5–15% SDS-polyacrylamide gradient gels, drying, autoradiography	Standard Laemmli procedure	[45]
30 μl	KGSTAENAEYLRV	1 m*M*	20 m*M* HEPES, 7.5	15 m*M* $MnCl_2$	150 m*M* NaCl, 0.1% Triton X-100, 10% glycerol	45 m*M*	15 μl 3×SDS sample buffer	Electrophoresis on 5–15% SDS gradient gels on a 20% SDS layer, drying, autoradiography	Standard Laemmli procedure	[44]
Hydrolysis and Ptyr analysis by high voltage paper electrophoresis[c]										
50 μl	DAEYAARRRG and derivatives	50 μg	50 m*M*, Tris, 7.5	10 m*M* $MgCl_2$	10 μ*M* vanadate, 0.05% Triton X-100	20 μ*M*	50 μl 5% TCA	Hydrolysis (6 *M* HCl), paper electrophoresis, counting	(e) Formic acid–acetic acid–water (25:78:897) pH 1.9, 150 V/cm, 150 min	[109]
Phosphocellulose paper[d]										
50 μl	DAEYAARRRG	50 μg	50 m*M*, Tris 7.5	10 m*M* $MgCl_2$	10 μ*M* vanadate, 0.05% Triton	20 μ*M*	50 μl 5% TCA	Washings, drying, counting	(w) 4 times with 0.5% phosphoric acid	[91]
100 μl	DRVYIHPF	3 m*M*	20 m*M* HEPES, 7.4	5 m*M* $MgCl_2$, 5 m*M* $MnCl_2$		50 μ*M*	500 μl 10% TCA	Washings, drying, counting	(w) 3 times with 1% phosphoric acid	[110]
50 μl	DRVYIHPF	2 m*M*	25 m*M* Tris, 7.5	10 m*M* $MgCl_2$		10 μ*M*	10 μl 0.5 *M* EDTA	Soaking in 30% ice-cold TCA, washings, drying, counting	(w) 6% acetic acid	[20,89]
15 μl	DRVYVHPF	1 m*M*	50 m*M* Tris, 7.6	10 m*M* $MgCl_2$, 15 m*M* $MnCl_2$	10 μ*M* vanadate, 0.1% NP-40	10 μ*M*	25 μl 4.1% TCA, 10 μg BSA	Washings, drying, counting	(w) 5 times with 0.5% phosphoric acid	[111]
?	DRVYIHPF	1 m*M*	20 m*M* HEPES	2 m*M* $MnCl_2$, 5 m*M* $MgCl_2$	50 μ*M* vanadate, 0.2 μ*M* $HS(CH_2)_2OH$, 24 μ*M* EDTA, 0.5% aprotinin, BSA (0.5 mg/ml)	5 μ*M*	5% TCA (final)	Washings, drying, counting	(w) 4 times with 75 m*M* phosphoric acid	[112]
25 μl	DRVYVHPF among others	50–5000 μ*M*	80 m*M* Tris, 7.5	20 m*M* $MgCl_2$	100 μ*M* vanadate, 14 m*M* pNPP, 0.8 mg/ml BSA	400 μ*M*	60 μl 20% TCA	Washing, drying, counting	(w) 0.5% phosphoric acid with 0.25% NaPP	[60]
30 μl	DRVYVHPF	1 m*M*	50 m*M* HEPES, 7.5	10 m*M* $MnCl_2$	10 μ*M* vanadate	24 μ*M*	50 μl 5% TCA + 20 μl 1% BSA	centrifugation, washings, drying, counting	(w) 4 times with 0.5% phosphoric acid	[113]
25 μl	DRVYVHPF among others	1 m*M*	50 m*M* Tris, 7.7	10 m*M* $MnCl_2$	1.2 mg/ml BSA, 100 μ*M* vanadate, 14 m*M* $HS(CH_2)_2OH$		45 μl 5% TCA + 3.5 m*M* ATP	Centrifugation, washings, drying, counting	(w) 6 times with 0.425% phosphoric acid, once with acetone	[114]
50 μl	DRVYVHPF	3 mg/ml	50 m*M* Tris, 7.0	50 m*M* $MgCl_2$	50 μ*M* vanadate, 7 mg/ml pNPP	100 μ*M*	45 μl 67% TCA + 20 μl 30 mg/ml BSA	Washings, drying, counting	(w) 4 times with 0.3% phosphoric acid, once in acetone	[115]
50 μl	DRVYVHPF		150 m*M* HEPES, 8.0	6 m*M* $MgCl_2$, 6 m*M* $MnCl_2$	3 m*M* $HS(CH_2)_2OH$, 10% glycerol, 0.01% Triton X-100	200 μ*M*	Spotting on paper	Washings, drying, counting	(w) 75 m*M* phosphoric acid	[85]
40 μl	RRKGSTAANAAYLRV and derivatives	750 μ*M*	12.5 m*M* HEPES, 7.4	10 m*M* $MnCl_2$	50 μ*M* vanadate	10 μ*M*	17 μl 24% TCA + 100 μg BSA	Centrifugation, washings, drying, counting	(w) 4 times with 75 m*M* phosphoric acid, once in acetone	[61]
50 μl	EKIGEGTYGVVYK and derivatives	300 μ*M*	50 m*M* Tris, 7.0	50 m*M* $MgCl_2$	2 m*M* DTT, 40 m*M* NaCl	100 μ*M*	Spotting on paper	Washings, drying, counting	(w) 4 times with 0.3% phosphoric acid, once in acetone	[53]
30 μl	TRDIYETDYRK	2 m*M*	50 m*M* HEPES	5 m*M* $MnCl_2$		25 μ*M*	50 μl 5% TCA + 20 μl 1% BSA	Centrifugation, washings, drying, counting	(w) 4 times with 75 m*M* phosphoric acid	[116]
25 μl	EEEEYMPME and DRVYIHPF		20 m*M* HEPES, 7.4	5 m*M* $MnCl_2$	10 m*M* thioglycerol	10 m*M*	1 m*M* cold ATP	Washings, drying, counting	(w) 10% TCA	[49]
30 μl	RRLEEEEEAYG	2 m*M*	20 m*M* HEPES, 7.4	2 m*M* $MnCl_2$	0.2% NP-40, 10 μ*M* zinc acetate	50 m*M*	7.5 μl 50% TCA	Washings, counting	(w) Acetic acid	[59]

Table 1 (*continued*)

Volume	Typical peptide structure and concentration		Buffer, pH	Cation	Adjuvant(s)	ATP	Stop	Post-assay conditions	Buffers[c]	Refs.
30 μl	RRLIEDNEYTARG	2 m*M*	20 m*M* HEPES	2 m*M* $MnCl_2$	0.2% NP-40, 10 μM zinc acetate	50 m*M*	50 μl 5% TCA+20 μl 1% BSA	Washings, counting	(w) Acetic acid	[117]
30 μl	DRVYIHPF	1 mg/ml	50 m*M* PIPES, 7.5	5 m*M* Mg^{2+}	0.1 m*M* EDTA, 0.015% Brij 35, 0.1 mg/ml BSA, 0.2% $HS(CH_2)_2OH$	0.1 m*M*	120 μl 10% phosphoric acid	Washings, drying, counting	(w) With 6% acetic acid, once in acetone	[58]
??	RRLEEEEEAYG	0.2 m*M*	20 m*M* imidazole, 7.4	12 m*M* $MgCl_2$, 2 m*M* $MnCl_2$	100 m*M* NaCl, 5% glycerol, 0.02% Triton X-100, 10 m*M* pNPP, 100 μM vanadate	200 μM	30 μl 5% TCA	Washing, drying, counting	(w) 5 times with 75 m*M* phosphoric acid.	[118]
100 μl	KHKKLAEGSAYEEV	200 μM	25 m*M* HEPES, 7.4	5 m*M* $MgCl_2$, 2 m*M* $MnCl_2$	50 m*M* vanadate	10 m*M*	2 ml 75 m*M* phosphoric acid	Washings, counting	(w) 5 times with 75 m*M* phosphoric acid	[25]
50 μl	EDAEYAARRG among others	1 m*M*	50 m*M* Tris, 7.8	50 m*M* Mg^{2+}	10 m*M* vanadate	60 m*M*	150 ml 3.3% TCA+10 ml 20 mg/ml BSA	Centrifugation, washings, drying, counting	(w) 4 times with 0.5% phosphoric acid, once with acetone	[119]
Centrifugation, substrate on resin beads[b]										
50 μl	RDIYETDYYRKGG among others	2 m*M*	50 m*M* HEPES, 7.4	4 m*M* $MnCl_2$	0.1% Triton X-100	50 μM	50 m*M* NaF, 10 m*M* NaPP, 5 m*M* EDTA, 5 m*M* EDTA	Centrifugation, washings, counting	(w) 3 times with 40 m*M* phosphate and 5% SDS, once with 5% SDS	[105]
Substrate on resin beads[b]										
100 μl	All possible heptapeptides	500 000 beads	30 m*M* MES, 6.8	10 m*M* $MgCl_2$, 0.4 mg/ml BSA		100 μM	washing NaCl, KCl, Tween 20	Poured in 1% agarose on plates, autoradiography, excision, sequencing	None	[102]

[a] The method is adapted from Ref. [120] for the ion-exchange procedure and from Ref. [38], for the molybdate treatment.

[b] This method can be used when peptidic substrates are searched inside peptide libraries.

[c] Originally described by Meggio et al. [121].

[d] The method has been initiated by Glass et al. [56]; serine/threonine protein kinase assays after observation by Witt and Roskoski [55].

[e] (w) washing buffer, (e) elution buffer, (a) electrophoresis buffer, and (t) TLC buffer.

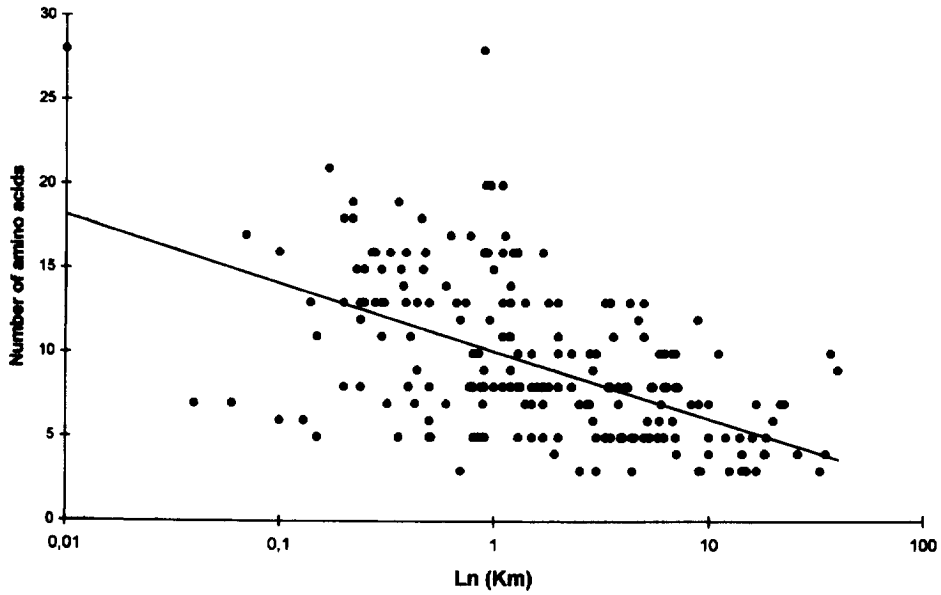

Fig. 1. Relationship between the length of the TPK peptide substrate and its K_m. Adapted from Ref. [11].

2. Affinity measurement of specific types of molecules to kinase, e.g., the binding of phorbol ester derivatives to protein kinase C, a serine/threonine protein kinase.
3. Detection of the recently discovered tight association of the tyrosine-phosphorylated SH2 regions with specific proteins or model peptides [30,34]. A quantification of this important process is in progress [35].
4. Enzymatic assay of TPK activity which would include enzyme, substrate, [^{32}P]γ-ATP, divalent cations and several ingredients more specific of the particular TPK (see list in Table 1). The analysis of the reaction product after the incubation is the real difficulty. The product of the reaction is either visualised, using a spectrometric technique or quantified "blindly" either after being physically linked to a solid support through covalent (beads) or ionic bonds (phosphocellulose paper) or after a combination of extractions (liquid–liquid or SPE). In the first case, the phosphopeptide may be positively recognised and therefore the quality of the separation from the labelled ATP is not crucial. In contrast, in the second approach performed without a positive recognition step, it is possible to have a contamination of the phosphopeptide due to partial failure of its separation from non-reacted ATP.

2.2. Possible ways to assay TPK

TPK assays can take advantage of monitoring either substrate modification or co-substrate(s) consumption (ATP or GTP). Surprisingly, to my knowledge, an assay based on the consumption of ATP to measure TPK activity has not been described to date. Nevertheless, at least three methods would allow to reach this goal: an enzyme-cascade procedure linked to the transformation of ATP to ADP (see, e.g., Ref. [36] for UDPGT and Ref. [37] for alkaline phosphatase), an extraction procedure that permits to "specifically" extract labelled ATP from the incubation medium [38] and our own method, which takes advantage of the high affinity of a stationary phase (polyHYDROXYETHYL, PolyLC, Columbia, MD, USA) for ATP [39]. This last method has permitted to selectively and quantitatively clean the incubation medium from any traces of ATP, a feature that we applied to ATPase activity measurement [40]. This particular method, used as a SPE approach for the measurement of TPK activity, enabled us to count

Table 2
Phosphorylation of angiotensin and analogues by different tyrosine protein kinases.

Method	Peptide	Kinase	K_m (mM)	Reference
Chelation/ion-exchange chromatography	DRVYVHPF	c-*src*	0.9	[50]
Chelation/ion-exchange chromatography	DRVYVHPF	c-*src*	0.8	[122]
Chelation/ion-exchange chromatography	DRVYVHPF	c-*src*	1.5	[85]
Ion-exchange chromatography/extraction	DRVYIHPF	TPK-IIA	1.6	[123]
Ion-exchange chromatography/extraction	DRVYIHPF	TPK-IIB	2.0	[48]
Ion-exchange chromatography/extraction	DRVYIHPF	*lyn*	1.8	[48]
Ion-exchange chromatography/extraction	DRVYIHPF	TPK-III	1.2	[48]
RP-HPLC	DRVYIHPF	HPK40	0.54	[28]
SDS gel electrophoresis/autoradiography	DRVYIHPF	EGF rec TPK	6.4	[45]
Paper electrophoresis	DRVYIHPF	TPK-IIB	0.77	[109]
Paper electrophoresis	DRVYIHPF	TPK-III	1.0	[109]
Paper electrophoresis	DRVYIHPF	v-*src*	2.0	[43]
Paper electrophoresis	$RVYIHPF	v-*src*	4.2	[43]
Paper electrophoresis	DRVYVHPF	v-*src*	0.24	[124]
Paper electrophoresis	DRVYVHPF	v-*src*	0.87	[43]
Paper electrophoresis	DRVYVHPF	Y73-P90	1.1	[43]
Paper electrophoresis	DRVYVHPF	FSV-P140	1.7	[43]
Paper electrophoresis	DRVYVHPF	A431 membranes	1.3	[43]
Phosphocellulose paper	DRVYIHPF	TK-II	0.77	[91]
Phosphocellulose paper	DRVYIHPF	TK-I	2.0	[91]
Phosphocellulose paper	DRVYIHPF	c-*src*(?)	2.3	[110]
Phosphocellulose paper	DRVYIHPF	c-*src*	2	[89]
Phosphocellulose paper	DRVYIHPF	$p93^{fes/fps}$	3.5	[110]
Phosphocellulose paper	DRVYIHPF	TKI	0.87	[111]
Phosphocellulose paper	DRVYIHPF	Erythrocyte cytoplasmic TPK	3.8	[125]
Phosphocellulose paper	DRVYIHPF	Erythrocyte membrane TPK	3.4	[125]
Phosphocellulose paper	DRVYIHPF	TKII	1.68	[111]
Phosphocellulose paper	DRVYIHPF	EGF rec TPK	0.8	[112]
Phosphocellulose paper	DRVYVHPF	v-*src*	0.9	[60]
Phosphocellulose paper	DRVYVHPF	Platelet cyto TPK	1.1	[113]
Phosphocellulose paper	DRVYVHPF	HeLa p94 kinase	0.40	[114]
Phosphocellulose paper	DRVYVHPF	Spleen kinase	5.4	[115]

Abbreviations: $, sarcosine; TPK, tyrosine protein kinase.
Adapted from Ref. [11].

the labelled peptide free from contamination by labelled ATP [41].

Elsewhere, in a TPK specificity survey more than 200 different peptide sequences from 24 down to 3 amino acids in length have been reviewed [11]. Interestingly, although these results came from 44 different publications, only a handful of different techniques were used. In fact, there are far more than 10 assays for measuring tyrosine phosphorylation. Table 1 gathers 37 different assays usable with short peptide structures. Twenty of these are variations of the phosphocellulose paper assay, as discussed below (see Section 3.3).

The rest of the methods used in TPK assays are related more to the nature of the substrate and can be divided roughly in two categories: those using labelled ATP and those using non-radioactive materials. The latter methods take advantage of the highly specific anti-phosphotyrosine antibodies which are now commercially available. Although these particular methods have been shown to be extremely powerful, they have two main limitations: the high cost, especially in large screening and purification processes, and the requirement of an immobilised substrate to permit quantification. They were essentially applied when the substrates were either proteins or synthetic polymers such as Poly(Tyr-Glu).

3. Peptidic assays

Table 1 gives the assay conditions for measuring TPK activity with short peptidic sequences. The description of the assay conditions, the various

concentrations of the ingredients, an indicative concentration in peptidic substrates and finally the means by which the assay is stopped are gathered in this table. The assay itself, i.e., the post-assay treatment that ultimately permits one to obtain the phosphorylation rate is then briefly described. On the whole, a surprisingly large variety of assay conditions is observed. In Table 1, assays using different types of tyrosine protein kinases are gathered. Variations in the composition of the incubation media are due to the nature of the TPK measured, for instance, the particular requirement for high salt concentration, or the dependence on the ligand of the receptor-associated TPK, such as insulin or EGF.

3.1. Electrophoretic assays

Among the methods listed in Table 1, thin-layer electrophoresis [42] has the advantage to be usable with a large number of peptidic structures, and therefore to be a tool for specificity studies. Unfortunately, the sequence of the peptide has a non-negligible influence on its own migration in an electric field. Therefore, when using different peptides derived from the Src autophosphorylation domain (KLIEDNEYTAR), changes must be made in the pH of the electrophoresis, once again to nicely separate the phosphorylated peptide from the non-reacted ATP, prior to the revelation step which is autoradiography (followed if necessary by aspiration of the cellulose from the plate, elution at pH 4.7 from the cellulose material and counting of the eluate for quantification). Nevertheless, the apparatus used for the method is not common in many laboratories. This particular method has been used, though, by other laboratories for specificity studies. Another electrophoresis method, on paper, this time, was proposed by Wong and Goldberg [43]. In the same work the classical phosphocellulose paper assay was used to check the results obtained with the electrophoretic method. Similarly, when these authors used other substrates (derived from the autophosphorylation site of $p60^{src}$), they used the electrophoretic method described by Hunter [42] (also see above). Therefore, it seems that the same type of limitations exists in both these electrophoretic methods: the separation of the phosphorylated peptide from the unreacted ATP requires prior calibration for each individual peptide, since the migration is heavily dependent on the net charge of the peptide, which in turn depends on the variations of the sequence.

Honegger et al. [44], as well as Hsu et al. [45] described assays based on SDS-gel electrophoresis. This separation technique takes advantage of the differences in size of the components to be separated. According to the theory, though, these molecular species are no longer separated according to their respective charges. Therefore, it could be anticipated that any type of peptide could be easily separated from low molecular masses (i.e., ATP). Unfortunately, although this is true for rather large peptides and proteins, the method is not adequate for small peptides (heptapeptides or smaller). Indeed, Honegger et al. [44], as well as Hsu et al. [45] used large peptides (13 and 17 amino acids, respectively), showing better adequacy of the method in these cases.

3.2. Chromatographic assays

For small peptides, as listed in Table 1, the use of chromatography under different modes was attempted by several authors. Kemp et al. [46] were pioneers in using many methods for the study of protein kinase specificities. They assessed a method in which serine/threonine protein kinase peptidic substrates were subjected after incubation to chromatography on ion-exchange material (AG) and rapidly eluted from the column by 30% acetic acid. The unreacted, labelled ATP remained bound to the column during this treatment. This simple method was adapted to the tyrosine protein kinase domain, but with some modifications, including an additional extraction step (isobutanol–benzene) plus a molybdate complexation step in order to totally clean the milieu from any residual traces of unreacted ATP [38,47–49]. This initially quite simple and straightforward method ended up in an heavy, multi-step technique. Despite this, numerous results were obtained from this group on the specificity of a series of purified TPKs. A variation of the same approach has been published by Budde et al. [50] in which the ion-exchange step took place on PEI cellulose, a minute variation of the previous technique. Interestingly, it can be expected that either simple HPLC methodology after the ion-exchange step, as we described [51] or a SPE step

using disposable cartridges filled with an hydrophilic chromatography phase [39] would render the methodology slightly less heavy. Other HPLC methods were also described [52–54]. HPLC could make one's life easy, at least with regard to the practical aspect of peptide phosphorylation analysis, although this technique has to be completed by an expensive piece of equipment for on-line detection of the radiolabelled peptides. For this reason HPLC did not emerge as in other fields as a major breakthrough in phosphorylation studies. For instance, Cheng et al. [53] used HPLC and detected the radiolabelled peptides by scintillation counting of the collected peaks. This tedious method is rather expensive and impairs one to do a large number of experiments (at least because it is time-consuming).

Also time-consuming is the method we described for the angiotensin derivatives [51]. Nevertheless, it offers the possibility to treat literally hundreds of samples at a time, by using automatic injectors and on-line Cerenkov detections. One run took about 35 min, including re-equilibration, and 48 h non-stop treatment of the samples did not cause any noticeable stability problems. Nevertheless, the method was extremely column-consuming, probably because of the use of large amount of TBAP, a counter-ion that permits to eluate ATP in the void volume. We believed, at that time, that a "universal" method could be found with HILIC, a hydrophilic chromatographic mode in which ATP tightly binds to the column or the cartridge, if one uses it as a pre-analysis treatment [39]. This method offered the possibility to test any peptide phosphorylation, from dipeptides up to the full Src-autophosphorylation sequence [41]. The only peptide (out of nearly 200) of which the phosphorylation could not be analysed was the mini-gastrin derivative LQQQQQAYG, suggesting that the method is largely applicable.

Another "universal" method [52] takes advantage of the extraordinary analytical potential of HPLC coupled to electrospray-mass spectrometry detection (ESMS). This purely analytical method does not require the use of labelled ATP. Furthermore, the necessary amount of phosphorylated peptide can be extremely low, thus compensating for the possible lack of sensitivity due to the absence of radioactive signal. Obviously, the main disadvantage is the cost of the equipment and the high technical requirement of this type of approach. Another potential problem is the nature of the eluant during LC separation immediately prior MS detection. The eluants used in these systems are often aqueous systems with small amounts of organic solvents, which considerably limits the potential of the technique (although modern instruments can handle up to 80% water but still have problems with inorganic buffers). A possible solution for this is again the use of hydrophilic chromatography in which the eluant is a solution of acetonitrile with small amounts of aqueous triethylamine–phosphoric acid (pH 2.8) [39].

3.3. Phosphocellulose assays

Historically, the phosphocellulose paper assay by far has been the most popular. First described by Witt and Roskoski [55], it has been particularly studied by Glass et al. [56], and most of the later adaptations are derived from this particular work. Initially, it has been "imported" from the serine/threonine protein kinase domain, at a time where TPKs were barely described. Interestingly enough, though, it was meant to be limited to arginine- and lysine-containing basic peptides, such as the RRLIEDAEYAARG sequence, derived from the autophosphorylation site of $pp60^{src}$. However, many authors used this method less carefully and some of the data reported in the literature for non basic substrates might be questionable. Nevertheless, in view of the data gathered in Table 2, one can see that the evaluation of the kinase activity with angiotensin II as the substrate, but using various methodologies, led to similar K_m values in the 1 m*M* range. It is therefore apparent that the phosphocellulose paper method is well adapted to the screening of inhibitors (e.g., Ref. [57]) or the kinase purification process [58]. In this last case, interferences due to the presence of high salt concentration have been repeatedly observed [27,28].

More questionable were the results published by Baldwin et al. [59] on the study of the phosphorylation of gastrin or minigastrin derivatives (RRLEEEEEAYG), the work by Radziejewski et al. [60] (DRVYVHPF: angiotensin II) or Klingbeil et al. [61] (RRKGSTEEEAEFLRV and other analogues of a sequence around Tyr 1173 of the EGF receptor), all using this particular phosphocellulose paper assay.

Table 3
Tyrosine protein kinase asays using protein or poly(Tyr-Glu) copolymers as substrate

Assay procedure	Pretreatment technique	Substrate	Reference
Electrophoresis, autoradiography	TPK immobilized on Sephadex beads through Ab	Enolase	[126]
Electrophoresis, autoradiography	In solution	"Glycolytic enzymes"	[66]
Electrophoresis, autoradiography	In solution	Co-purified proteins	[69]
Electrophoresis, autoradiography	In solution after immuno-precipitation of the kinase	Enolase	[127]
Electrophoresis, autoradiography	In solution after immuno-precipitation of the kinase	p36	[128]
Electrophoresis, autoradiography	In solution	Ab	[129]
Electrophoresis, autoradiography	In solution	RCAM-lysozyme	[130]
Electrophoresis, Western blotting, autoradiography	In solution	p44mpk	[8]
Electrophoresis, alkali treatment, autoradiography	In solution	Endogenous protein	[119]
Electrophoresis, NaOH hydrolysis, autoradiography	Immunoprecipitation of TPK	Enolase	[131]
Autoradiography	TPK immobilized in non-denatured electrophoretic gels	Poly(Tyr-Glu)	[132]
Autoradiography	Poured in polyacrylamide gel	Poly(Tyr-Glu)	[133]
Immunoprecipitation, electrophoresis, autoradiography	In solution	Phospholipase C-g	[134]
Immunoprecipitation, electrophoresis, excision, counting	In solution	IgG heavy chain	[135]
Precipitation by TCA, electrophoresis, excision, counting	In solution	Histones	[136]
Precipitation by TCA, electrophoresis, autoradiography	In solution	Among others, casein	[137]
Precipitation by TCA, washings, counting	In solution	Casein	[93]
Precipitation by TCA, washings, counting[a]	In solution	Poly(Tyr-Glu)	[22]
Precipitation with TCA, washings, counting	In solution	Poly(Tyr-Glu)	[93]
Precipitation with TCA, washings, counting	In solution	Poly(Tyr-Glu)	[92]
Precipitation with TCA, washings, counting	In solution	Poly(Tyr-Glu)	[91]
Acid precipitation, spotting on 3MM paper, counting	Immunoprecipitation of TPK	Poly(Tyr-Glu)	[138]
Phosphocellulose assay	In solution	Poly(Tyr-Glu)	[65]
Spotting on Whatman paper, counting	In solution	Poly(Tyr-Glu)	[86]
Spotting on Whatman paper, counting	In solution	Poly(Tyr-Glu)	[85]
Spotting on Whatman paper, washings, counting	In solution	Poly(Tyr-Glu)	[88]
Spotting on Whatman paper, counting	In solution	Poly(Tyr-Glu)	[87]
Spotting onto Whatman P81 paper, wash 5 times with 6% TCA, drying, counting	In solution	Casein	[89]
Spotting onto Whatman filter paper, wash with 10% TCA with 0.01 *M* sodium pyrophosphate, extraction with alcohol/extraction with ether, drying, counting	In solution	Among others, histone	[90]
APPTAb/perox-labeled 2nd Ab	Coated to microtiter plate wells[b]	Poly(Tyr-Glu)	[139]
Dot-blot on PVDF membranes/APPTAb/Immunogold silver staining	In solution[b]	Poly(Tyr-Glu)	[140]
APPTAb/perox-labeled 2nd Ab	Coated to microtiter plate wells[b]	Poly(Tyr-Glu)	[141]

[a] Derived from the protamine kinase assay [94].
[b] Non-radioactive methods.

Toomik et al. [62] published an interesting work in which they checked the reliability of this assay. Referring to six different groups using this particular method, but with various substrates (HRASV, RRASA, Gva-RASV, LRRASVR, FRRLSI and LRRASLG, as well as the commercially available reference peptide RRLIEDAEYAARG), they demonstrated that the reliability of the assay was poor, since after phosphorylation 7–85% of these phosphorylated peptides did bind to the phosphocellulose paper, depending on their sequence and concentration. In another specificity study, Garcia et al. [63] carefully chose candidate substrates (1) by using rather large peptides with minute variations in their sequences, without touching (2) the two basic residues at the N-terminus of the peptides. This precaution, though, might have been insufficient in some cases where the overall charge was neutral to acid, and therefore could not guarantee a satisfactory binding of the peptide onto the phosphocellulose paper. The problem, despite these clear observations, still seems to be very underevaluated in the litera-

Table 4
Some of the tyrosine protein kinase autophosphorylation assays

Technique	Tyrosine protein kinase	Reference
Purification/phosphorylation/gel electrophoresis/autoradiography	TyrK 1 and TyrK II	[86]
Phosphorylation on immunocomplex/gel electrophoresis/KOH treatment/autoradiography	*c-neu*	[142]
Phosphorylation on immunocomplex/gel electrophoresis/Western blot/KOH treatment/autoradiography	$p60^{v-src}$	[143]
Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	p140 FSV	[144]
Purification/phosphorylation/gel electrophoresis/autoradiography	$p150^{abl}$	[58]
Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	*src*	[127]
Immunoprecipitation/phosphorylation/gel electrophoresis/excision of the bands/counting	EGF receptor protein kinase	[44]
Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	$p210^{bcr-abl}$	[97]
Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	PDGF receptor protein kinase	[145]
Purification/phosphorylation/gel electrophoresis/autoradiography	*src*	[89]
Solubilization/gel electrophoresis/Western blot/APPTAb/^{125}I-labelled secondary Ab/autoradiography	EGF receptor protein kinase	[146]
Immunoprecipitation/phosphorylation/gel electrophoresis/Western blot/APPTAb	*ros*	[147]
Purification/phosphorylation/gel electrophoresis/autoradiography	Insulin receptor protein kinase	[116]
Immunoprecipitation/phosphorylation/gel electrophoresis/KOH treatment/autoradiography	*fms*	[148]
Purification/Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	$p56^{lck}$	[149]
Purification/phosphorylation/gel electrophoresis/autoradiography	$p42^{mapk}$	[150]
Immunoprecipitation/phosphorylation/gel electrophoresis/autoradiography	*syk*	[151]
Purification/phosphorylation/filtration through Durapore filters/washings/counting	$p56^{lck}$	[152]

ture, even in the excellent book on protein phosphorylation [64], in which the assay is warningless given as trustworthy.

Finally, the few last methods listed in Table 1 are those developed in the particular context of peptide libraries (see Section 6).

4. Protein assays

4.1. Proteic versus peptidic assays

As stated in the Section 1, it is tempting to use large proteins, or artificial ones built for this precise purpose, e.g., the polymers of tyrosine and glutamic acid (poly(Tyr-Glu) [65]), to study the enzymological properties of protein kinases. In recent years, this particular approach has been preferred, because of the intuitive idea that protein kinases prefer proteins as substrates since they have evolved to do that kind of job. An early observation by Cooper et al. [66] showed that enzymes such as enolase and lactate dehydrogenase were good substrates for tyrosine phosphorylation in vitro, as well as in vivo. A systematic search for such endogenous substrates of $p60^{src}$ has been attempted [67] with specific antiphosphotyrosine antibodies immobilised into a column through which the phosphorylated proteins were chromatographed. Selective elution of the bound proteins (i.e., those which where phosphorylated on tyrosine residues) was obtained by applying a buffer containing 5 m*M* phosphotyrosine (see also Ref. [68] for p22, another substrate of $p60^{src}$). It is clear, though, that "new" proteic substrates such as those we found in HL60 [69], have a particular interest. Since they are co-purified with the target tyrosine protein kinase [69] they seem to be natural substrates although their nature and role are still unknown.

4.2. Difficulties related to the use of proteic substrates

At this point the following questions might be raised. Does phosphorylation occur on tyrosine only? Are the natural sites of the natural protein substrates already fully phosphorylated, masking the de novo

phosphorylation during the assays? Experimental evidences to answer the first question do exist. There are several techniques to analyse phosphorylated amino acids in the protein hydrolysate [70–77]. Most of these techniques have a derivatization step and a determination of the amount of phosphohydroxyamino acid using a protein sequencer. Alternatively, methods have been described for the measurement of phosphate content of the protein hydrolytes [74,75]. All of these techniques are difficult to handle but the evidences they bring are essential for the understanding of the phosphorylation mechanism. The technical limitation due to possible phosphorylation of serine and threonine beside tyrosine can be overcome by a simple treatment of SDS gels: only tyrosine-phosphorylated proteins resist incubation in KOH for 2 h at 60°C [79]. Furthermore, Bourassa et al. [80] modified the initial method in order to limit the slow release of proteins from the gel during alkali treatment.

The role of phosphorylated tyrosine in recognition of particular sequences [30,81–83] might also deeply modify the apparent result of in vitro phosphorylation assays, since association of pre-phosphorylated regions of the proteic substrates is favoured within the SH2 domain of TPK [34,84]. As pointed out elsewhere [11], the use of a substrate bearing several putative phosphorylation sites can be misleading. In fact, pre-phosphorylation allosterically facilitates further phosphorylations on other sites of a given substrate [78].

As a substitute of pure protein substrate, poly(Tyr-Glu) is nowadays a rather standard substrate to study TPK activities. It tends to solve two problems: the availability of the substrate and the absence of serine or threonine phosphorylation sites. Nevertheless, two major weaknesses have to be considered: (1) it is not a single chemical species, but rather a mixture of polymers of different lengths and therefore it does not behave as a pure protein (i.e., a single band on an SDS gel) and (2) it is an artificial substrate, lacking any type of motif that could participate to a substrate/enzyme recognition sequence.

4.3. Protein substrate assays: the list

Some of the techniques used in studies of polymer and protein phosphorylation are listed in Table 3. Most of the assays implies rather standard SDS-PAGE separation steps followed by autoradiography.

The use of natural proteins as substrates is important and the technical difficulties are less numerous than with peptidic substrates. The use of labelled ATP is no longer an absolute requirement. It can be replaced by cold ATP and the ultimate recognition test will be done with anti-phosphotyrosine antibodies.

The assays used are based on three kinds of techniques: electrophoresis, acid precipitation and immunological methods (Table 3). As stated for the peptide-based assays, the use of the phosphocellulose assay using protein as substrate is surprising. Indeed, the phosphocellulose-based assay requires several basic residues to permit the fixation of the substrate on the solid-phase (see above). The use of poly(Tyr-Glu) as substrate [65] is theoretically impossible, since this co-polymer totally lacks basic residues. Alternatively, this copolymer can be used when acid precipitation is followed by filtration on solid support [84–88], the size of the polymer granting its effective separation from labelled ATP of smaller molecular mass. Miscellaneous proteins have also been used with this SPE-type technique [89,90].

A simpler approach is to acid-precipitate the protein (or the co-polymer) after phosphorylation and, subsequently, wash the precipitate with TCA solutions. This particular technique proved to be extremely easy for the assessment with various types of substrates, especially the poly(Tyr-Glu) co-polymer [22,91,93], a method we derived [22] from the protamine kinase assay [94]. Ultimately, immunological methods using an antiphosphotyrosine antibody-based technique has been applied to the detection of the phosphorylation of poly(Tyr-Glu). This simple technique, although probably costly, has the advantage that it allows the use of non-radiolabelled ATP, a safety feature that should not be underestimated.

5. Autophosphorylation

This particular method allows access to other types of information concerning the functioning of receptors or other tyrosine protein kinases at the molecular level. It also has been used for the

Table 5
List of peptides used as tyrosine protein kinase substrates (peptides in alphabetical order)

Peptide substrate	Ref.	Peptide substrate	Ref.	Peptide substrate	Ref.	Peptide substrate	Ref.
AARQfPDNEY-cyclic	[122]	EIYEEYE	[159]	KKSRGDYITMQIG	[63]	RRKGSTAEEAEYLRV	[61]
AAVPSGASTGIYEALELR	[66]	EKEYH	[47]	KKSRGDYMTMQIG	[63]	RRKGSTAENAAYLRV	[61]
AAYAA	[48]	EKEYHAE	[47]	KKSRGDYMTTQIG	[63]	RRKGSTAENAESLRV	[61]
ADPDHDHTGFLTEYVATRWRR	[8]	EKIGEGTYGVVYK	[53]	KKSRGDYTTMQIG	[63]	RRKGSTAENAEYLRV	[61]
AEEEIYGEFEAKKKK	[153]	ELPYAG	[122]	KLDNEYTAR	[42]	RRKGSTAENAEYLRVAPQSS	[61]
AEEEYFFLF	[30]	ELPYAG-cyclic	[122]	KLIDNEYTAR	[42]	RRKGSTAENAEYLRVVAPKSS	[61]
AEVIYAAPF	[30]	ENYE	[41]	KLIEDNEYTA	[42]	RRKGSTAENAEYMNMAPQSS	[61]
AEYAA	[48]	Ac-ENYEIGLPPIIGK	[41]	KLIEDNEYTAR	[42]	RRKGSTEEEAEYLRV	[61]
AEYAARRG	[105]	EPQYQPA	[48]	KLLEEAEYLAREAAKF	[60]	RRKGSTEEEAEYMNMAPQSS	[61]
AEYTA	[48]	EYA	[155]	KQVVDS(iA)YEVIK	[158]	RRLDTTGQEEYSA	[59]
AFLEAFFTSTEPQYQPGENL	[154]	EYAA	[155]	KQVVDSAYEVIK	[158]	RRLEEEEEAYG	[59]
AFLEDFFTSTEPQYQPGENL	[154]	EYE	[41]	KRLIEDEYTARQGGC	[8]	RRLEEEEEAYG	[117]
AFLEDYFTSTEPQYQPGENL	[154]	EYH	[47]	KRSYEEHIPYTHMNGGK	[135]	RRLIADAEYAARG	[117]
AIYWHHY	[159]	EYHAE	[47]	KVEKIGEGSYGVVYK	[53]	RRLIEAAEYAARG	[117]
APGDRIYVHPF	[41]	EYSA	[41]	KVEKIGEGTYGVCYK	[53]	RRLIEDAEYAARRG	[112]
AREGSFEARYNNPFEDFRI	[45]	EYSAM	[41]	KVEKIGEGTYGVVKK	[53]	RRLIEDAEYARG	[135]
ARQIEDNEYA	[122]	*EAYG	[41]	KVEKIGEGTYGVVYK	[53]	RRLIEDAIYARG	[52]
ARQIEDNEYA-cyclic	[122]	*EAYL	[41]	KVEKIGEGTYGVVYK-NH_2	[53]	RRLIEDNEYTARG	[117]
AY	[123]	*EAYV	[41]	KVEKIGEGTYSVVYK	[53]	RSYEEHIPYTHMNGGKK	[105]
AYA	[123]	*EEEEEAYV	[41]	KVEKIGVGSYGVVYK	[53]	RVYIHPF	[43]
AYAA	[123]	*EEEYSAM	[41]	KVEKIGVGTYGVVYK	[53]	RVYIHPFHL	[41]
CADFGLARLIEDNEYTARG	[115]	*EEYE	[41]	KVETIGEGTYGVVYK	[53]	RVYIHPI	[41]
CEKIGEGTYGVVYK	[115]	*EGPWLEEEEEAYGWMDF	[112]	KYE	[41]	RVYVHPF	[41]
CSTPPSAYGSC-cyclic	[60]	*EHWSYGLRPG	[41]	LDDDDDAYV	[41]	RVYVHPI	[41]
Ac-DAEYA-NH_2	[153]	*ELYENK	[41]	LEDAEYAARRRG	[105]	$RVYIHPA	[41]
DAEYAAR	[93]	*ELYG	[41]	LEEEEEAYGWMDF	[41]	$RVYIHPF	[43]
DAEYAARRRG	[93]	*ELYGNKPR	[41]	LEEEEEAYVWMDF	[41]	$RVYIHPG	[41]
DAEYAARRRG	[105]	*EVYV	[41]	LEEEEEVYGWMDF	[41]	$RVYIHPI	[41]
DDFYVAV	[41]	GATYTAAA	[156]	LEEEEEVYV	[41]	$RVYIHPL	[41]
DDYSAV	[41]	GATYTAHA	[156]	LEEEEEVYVWMDF	[41]	$RVYIHPT	[41]
DEDYIQD	[41]	GAY	[155]	LEEEEVYV	[41]	$VYIHPI	[41]
DENYYKA	[154]	GAYWHHY	[159]	LEEEEYI	[41]	TRDIYETDYYRK	[135]
DFEPAEDYL	[41]	GDAYAAHA	[156]	LEEEVYV	[41]	TYS	[41]
DRVYIHP	[41]	GDTYTAAA	[156]	LEEVYV	[41]	VALFDYESR	[41]
DRVYIHPA	[41]	GDTYTAEA	[156]	LEYEL	[41]	VALYDFESR	[41]
DRVYIHPF	[91]	GDTYTAHA	[156]	LFASSNPEYLSARR	[135]	VALYDYESR	[41]
DRVYIHPFHL	[43]	GEG(Tp)YGV	[78]	LIEDAEYAARRRG	[105]	VYIHPF	[41]
DRVYIHPI	[41]	GEGTYGV	[78]	LIEDAEYTA	[157]	VYV	[123]
DRVYIHPT	[41]	Ac-GEGTYGV-NH_2	[153]	LIEDALYTA	[158]	WHHY	[159]
DRVYVHPF	[124]	GEGTYGVV	[41]	LIEDNEYTAR	[42]	(X)EEEYMMMM	[30]
DRVYVHPFHL	[41]	GIAWHHY	[159]	Ac-LPYA-$NHCH_3$	[153]	(X)E (X)IYGVLF	[30]
DRVYVHPFNL	[41]	GIYAHHY	[159]	LQDDYEDD	[41]	YA	[123]
DVGEYRAVTELG	[41]	GIYWAHY	[159]	LRRAYLG	[117]	YAA	[123]
DYE	[41]	GIYWHAY	[159]	MENYQKVEKIGEGTYGVVYKARHK	[53]	YAARFPEDNE-cyclic	[122]
DYM	[41]	GIYWHH	[159]	MNYLAFPRM	[41]	YEEEEE	[155]
EAYAA	[155]	GIYWHHA	[159]	N(Sp)YTA	[78]	YGGFLRKYPK	[41]

EDAAYAA	[48]	GIYWHHY	[159]	N(Tp)YTA	[78]	Ac-YGSFK	[160]
Ac-EDAEYA-NH_2	[153]	GGYR	[123]	NAYTA	[48]	YGSFK	[160]
EDAEYAARRG	[119]	GISEPYIE	[41]	NDYSA	[41]	YIYEEED	[159]
EDAEYAARRRG	[105]	GIYWHHY	[159]	NDYSAM	[41]	Ac-YIYGSFK	[160]
Ac-EDAIY-NH_2	[153]	GYA	[123]	NEYAA	[48]	YIY	[160]
EDNAYTA	[48]	HSDAVFTDNYTRLRKQMAVKKYLNSILN	[110]	NEYAAfPIED-cyclic	[122]	YIYASFK	[160]
EDNEYTA	[154]	IDDAAYTA	[158]	NEYE	[41]	YIYG	[160]
EDNEYTARQG	[43]	IEDAEYAARRRG	[105]	NEYT	[41]	YIYGAFK	[160]
EDNEYVARQG	[43]	IEDNEYTARQG	[117]	NEYTA	[155]	YIYGS	[160]
EEEAYGWMDF	[41]	IEEAAYTA	[158]	NIDGDGEVNYEE	[154]	YIYGSF	[160]
EEEEAYGWMDF	[41]	IENEEQEYVQTVK	[60]	NRVYVHPF*	[43]	yIYGSFK	[160]
EEEEEAYGWMDF	[41]	Ac-IYGEF-NH_2	[153]	NRVYVHPFHL	[41]	YiYGSFK	[160]
EEEEEY	[155]	Ac-IYGSFK	[160]	NRVYVHPFNL	[41]	YIyGSFK	[160]
EEEEYFELV	[30]	IYGSFK	[160]	NTYTA	[78]	YIYGsFK	[160]
EEEEYMPME	[49]	IYWHHY	[159]	NYYTA	[78]	YIYGSfK	[160]
EEEEYVFIE	[30]	KDDEYNPA	[154]	QEEYSAM	[41]	YIYGSFk	[160]
EEEIYEEIE	[30]	KEY	[123]	QEYSA	[41]	YIYGSFK	[102]
EEEIYGEFD	[30]	KEYH	[47]	QEYSAM	[41]	YIYGSYK	[160]
EEEVYV	[41]	KEYHAE	[47]	QEYSAV	[41]	YIYGTFK	[160]
EEEYEEE	[41]	KGSTAENAEYLRV	[44]	QNAAYAA	[48]	YIYPFK	[160]
EEIYGEFF	[30]	KGVYIHAL	[41]	RDIYETDYYRK	[33]	YIYβFK	[160]
EEKEYHAE	[47]	KHKKLAEGSAYEEV	[25]	RDIYETDYYRKGG	[105]	YIY$\beta\beta$FK	[160]
EENEYTA	[48]	KIEKIGEGTFGVVYK	[114]	RDRVYVHPF	[50]	YIYεFK	[160]
EEY	[155]	KIEKIGEGTYGVVFK	[114]	RGDEEIPEEYL	[41]	YQKVEKIGEGTYGVVYKARHK	[53]
EEYAA	[155]	KIEKIGEGTYGVVYK	[114]	RILSTYGRSAE	[41]	YVVKETIGVGSYSVCKRCVHK	[53]
EEYEE	[41]	KIGTAEPDYGALYEGR	[45]	RKGNGDGYMPMSPKSV	[63]	YWHHY	[159]
EEYSAV	[41]	KIYDDYE	[159]	RLIEDAEYTA	[158]	YYA	[123]
EEYVAV	[41]	KKGSEEYMNMDLGPGR	[63]	RLIEDALYTA	[158]	YYY	[123]
EEYVRFDSDVGE	[41]	KKHTDDGYMPMSPGVA	[63]	RLIEDNEYTARKG	[158]		
EGRNPGFYVEANPMPTFK	[45]	KKKEEEEEEYMPMEDL	[63]	RLIEDNEYTARNG	[158]		
Ac-EGTYG-NH_2	[153]	KKKLPATGDYMNMSPVGD	[63]	RLIEDNEYTARQE	[59]		
EGVYVHPV	[41]	KKRVDPNGYMMMSPSGS	[63]	RLMTGDTYTAHAGAK	[154]		
EIYEENG	[159]	KKSRGDY(Nle)TMQIG	[63]	RRKGSTAANAAYLRV	[61]		

Abbreviations: $, sarcosine; Nle, nor-leucine; β, β-alanine; ε, ε-amino caproic acid; *E, pyroglutamate; X, any of 15 natural amino acids (W, C, Y, S and T omitted) [30]; Tp, phosphothreonine; Yp, phosphotyrosine; Sp, phosphoserine. The lower case letters are used to depict the non-natural, d-amino acids.

screening of compounds with the aim to find inhibitors of the self-phosphorylation capacity of many receptors of growth factor. Indeed, autophosphorylation is the initial activation step of the pathway that ultimately leads to cell multiplication or transformation. Therefore, such a type of inhibition should permit the discovery of compounds that would impair this activation process. The techniques presented in Table 4 are simple and straightforward. Once the protein is isolated, phosphorylation takes place in similar conditions as for the kinase activity with exogenous substrates, and the phosphorylated product is isolated by electrophoresis and quantified for the incorporated radioactivity. Further evidences are sometimes provided concerning the nature of the site of autophosphorylation (tyrosine versus serine or threonine) by running other experiments (alkali treatment, antiphosphotyrosine antibody immunoassay, hydrolysis and phosphoamino acid analysis).

The reader is referred to a rather exhaustive list (however without technical details) of all the protein kinases and the corresponding assays, including autophosphorylation (Ref. [29] for tyrosine protein kinases and Ref. [95] for serine protein kinases).

Autophosphorylation of various TPK has been used in screening assays for the discovery of TPK inhibitors, e.g., herbimycin A [96], tyrphostins [97] and geldamycin [98]. For example, the experiments reported by Schnur et al. [98] describe the measurement of the phosphotyrosine content of the oncogene product $p185^{erbB\text{-}2}$ after treatment of infected cells with numerous geldanamycin derivatives.

6. Peptide libraries

The search for optimum substrates and inhibitors necessitates new approaches, particularly those providing large numbers of possible structures, since the traditional approach has not been successful in finding short and efficient peptides [11], despite the screening of more than 320 different peptides as substrate for TPKs (Table 5).

The synthesis of combinatorial peptide libraries forms a body of techniques that enable one to obtain mixtures of all possible peptides of a certain length build-up with either natural amino acids (phage techniques) or with any type of amino acids (chemical methods; for, review, see Refs. [99,100]).

6.1. Libraries in TPK substrate specificity studies

The first application to the search of kinase substrates was published by Till et al. [52]. These authors used the library technology to synthesise the analogues of Kemptide (LRRASLG) in which Arg at position 2 was replaced in a library (i.e., all the analogues mixed together) by all the other natural amino acids (C omitted). Similarly, they replaced in the Src-derived sequence RRLIEDAEYAARG, the Glu at position 8 by all the possible natural amino acids. These experiments were conducted to search for better substrate analogues for protein kinase A and v-*abl* tyrosine protein kinase, respectively.

A more recent work on protein kinase A with peptide libraries, provided a new substrate (Ac-RAERRASI, $K_m = 2\ \mu M$) amongst a population of 2.56×10^{10} octapeptides in libraries [101]. In another combinatorial work [102], the best substrate of $pp60^{src}$ to date ($K_m = 55\ \mu M$) could be fished out amongst all the possible heptapeptides synthesised from 19 building blocks (i.e., all the natural amino acids, cysteine omitted: 893×10^6 individual peptides). Within this population, the methodology identified only one structure: YIYGSFK as substrate of $p60^{src}$. Although questionable about the physical feasibility of such a number of peptides (as discussed elsewhere [103,104]), these particular methods open new routes to original and possibly more potent substrates of protein kinases (see also Ref. [52]). This approach was also implicit in the work of Chavanieu et al. [105], who described the first protein kinase assay using putative insulin receptor tyrosine protein kinase substrates immobilised on beads.

6.2. Library approach: technical challenge and solutions

From a technical point of view, the use of complex mixtures of putative substrates is raising special problems. According to certain types of peptide library generation, it is possible to test them while the libraries are still linked to the beads on

which they were synthesised thus neglecting the influence of the steric hindrance of the bead in enzyme/substrate recognition. Chavanieu et al. [105] demonstrated that the measurement of immobilised substrate phosphorylation by the insulin receptor-associated tyrosine protein kinase was possible, as previously demonstrated for other types of biological assays using these immobilised peptides. Because of the great number of putative substrates, another solution [52] is to analyse the incubation medium for the presence of all the phosphorylated peptides. For this particular case, the problem was simple since the tested libraries were limited to 19 analogues of a given peptide (RRLIEDAXYAARG in which X was one of the natural amino acids, C omitted), which permitted the quantification of the phosphorylation of all the analogues simultaneously. Nevertheless, this result required the use of a high-tech type of approach (LC–ESMS). Whenever larger libraries are screened, technical solutions do exist [106]. According to Songyang et al. [106] it is possible to screen billions of peptide libraries for the specificity of serine [106] or tyrosine protein kinases [30]. After a filtration step to separate the phosphopeptides from the proteins, two more steps are necessary (DEAE- and ferric chelation chromatography) to isolate the phosphopeptide. The final step is the identification of the phosphorylated peptide by sequencing [106]. This work provided the first systematic study to TPK specificity. Indeed, it described eight different peptides rather specific for eight different TPKs, and respective cross-selectivity is poor. Lam et al. [102] described a beautiful approach in which, after the phosphorylation in presence of labelled ATP and of Src kinase, the beads bearing the libraries were thoroughly washed and immobilised inside an agarose gel. The dark spots on the gel revealed by autoradiography, were excised and extracted from agarose. A single repetition of the autoradiographic screening in agarose was sufficient to ensure the excision of a single bead per dark spot. The corresponding beads were then inserted into a protein sequencer in order to determine the sequence of the substrate. As mentioned earlier, combinatorial libraries require new technical solutions and often expensive and sophisticated equipment. A simpler solution for this type of assay, such as the measurement of the consumption of ATP during incubation, may exist although it has not been tested for tyrosine protein kinase phosphorylation assays.

The relative new discovery of the high affinity of the SH2 domain of kinase with vicinal target protein has also been investigated with model peptides from synthetic libraries [81–83], as well as the exploration of the SH3 ligands, another important step in protein–protein recognition, using phage-displayed libraries [107]. There are already several studies published on the search of SH2 "inhibitors" [35] and the related chemistry [108].

7. Concluding remarks

There is a large number of techniques available to measure the activity of tyrosine protein kinases. They range from simple electrophoretic to chromatographic procedures to sophisticated LC–MS techniques. When the goal is to determine the specificity of the enzyme(s), another method will be preferred, e.g., the chelation and extraction of unreacted ATP or the SPE extraction of ATP by hydrophilic chromatography. These two methods have been successfully used with large families of peptides of various structure [41,47,48,78], both offering a good level of trustability for this particular type of research. For other purposes (purification, screening), a complete assessment of the phosphocellulose paper method with a carefully chosen peptide substrate should be done as advocated by Toomik et al. [62].

The techniques for the study of the functions of tyrosine protein kinases have been enriched with several sophisticated approaches that explore new areas like the role of the SH2 domains. Similarly, the screening of peptide libraries will soon provide completely new and hardly predictable leads for the synthesis of specific inhibitors. This approach is likely to be more straightforward and rational than the systematic screening of natural and synthetic compounds. These elegant studies far more complex than simple assays, will permit a precise dissection of the pathways leading to cell growth and cell transformation. They are, thus, amongst the most valuable advances in this domain closely related to cancer.

Assays are often considered as simple auxiliary tools, but as exemplified in the present review, they

play a key role for the identification of the enzymes of the tyrosine protein kinase family and the understanding of their respective specificities. They also play a major role in the design of screening processes in which new inhibitors will be found. Further progress improving their flexibility is a highly desirable goal.

8. List of abbreviations

ADP	Adenosine 5′-diphosphate
AMP	Adenosine 5′-monophosphate
APPTAb	Anti phosphotyrosine antibody
ATP	Adenosine 5′-triphosphate
ATPase	Adenosine 5′-triphosphatase
BSA	Bovine serum albumin
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(aminoethyl) ether
ESMS	Electrospray mass spectrometry
GTP	Guanosine 5′-triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]
HILIC	Hydrophilic chromatography
HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography coupled to mass spectrometry
LC–ESMS	Liquid chromatography coupled to ESMS
MES	2-[N-Morpholino]ethanesulfonic acid
NaPP	Sodium pyrophosphate
PEI	Polyethyleneimine
PIPES	Piperazine-N,N′-bis[2-ethanesulfonic]
pNPP	*p*-Nitrophenylphosphate
SDS	Sodium dodecyl sulfate
SH2	*src* homology region No. 2
SH3	*src* homology region No. 3
SPE	Solid-phase extraction
TBAP	Tetrabutyl ammonium dihydrogen phosphate
TCA	Trichloroacetic acid
TEAP	Triethylamine–phosphoric acid
TFA	Trifluoroacetic acid
TPK	Tyrosine protein kinase
Tris	Tris[hydroxymethyl]aminomethane
UDPGT	Uridine diphosphate glucuronosyl transferase

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