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NMR analysis of regioselectivity in dephosphorylation of a triphosphotyrosyl dodecapeptide autophosphorylation site of the insulin receptor by a catalytic fragment of LAR phosphotyrosine phosphatase

JONATHAN P. LEE,[1] HYEONGJIN CHO,[1] WILLI BANNWARTH,[2] ERIC A. KITAS,[2] AND CHRISTOPHER T. WALSH[1]

[1] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and the Dana Farber Cancer Institute, Boston, Massachusetts 02115
[2] Central Research Department, Hoffmann-La Roche, Basel, Switzerland

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Abstract

An autophosphorylation site in the activated insulin receptor tyrosine kinase domain has three tyrosines phosphorylated when fully activated. To begin to examine recognition of triphosphotyrosyl sites by protein tyrosine phosphatases in possible control of signal transduction a triphosphotyrosyl dodecapeptide TRDIpYETDpYpYRK corresponding to residues 1,142–1,153 of the insulin receptor was prepared and incubated with the 40-kDa catalytic domain of the human PTPase LAR. To assess regioselectivity of recognition, the three diphosphotyrosyl regioisomers, and the three monophosphotyrosyl regioisomers were prepared and assayed. All seven peptides were PTPase substrates. To identify any preferences in dephosphorylation at pY5, pY9, or pY10, ^{1}H-NMR analyses were conducted during enzyme incubations and distinguishing fingerprint regions determined for each of the seven phosphotyrosyl peptides. LAR PTPase shows strong preference for dephosphorylation first at pY5 (at tri-, di-, and monophosphotyrosyl levels). Initially this regioselectivity gives the Y5(pY9)(pY10) diphospho regioisomer, followed by equal dephosphorylation at pY9 or pY10 to give the corresponding monophosphoryl species on the way to fully dephosphorylated product. The NMR methodology is applicable to other peptides with multiple sites of phosphorylation that undergo attack by any phosphatase.

Keywords: LAR PTPase; leukocyte antigen related phosphotyrosine phosphatase; regioselective dephosphorylation

The balance between phosphorylation and dephosphorylation of specific tyrosyl residues in proteins mediates the strength and duration of signal transduction events in many growth factor-driven cell proliferative responses. These opposing signals are controlled by the activity and location of tyrosine kinases and PTPases. Both the src family of tyrosine kinases and the superfamily of receptor tyrosine kinases have their catalytic activity regulated by tyrosine phosphorylation at multiple sites (Kmiecik et al., 1988; Cantley et al., 1991). The IR has been a particularly well-studied case in which binding of insulin at the outside stimulates autophosphorylation of at least five tyrosine residues in the catalytic domain on the cytoplasmic side of the membrane (Tornqvist et al., 1987; White et al., 1988; Murakami & Rosen, 1991). Three of these tyrosines at residues 1,146, 1,150, and 1,151 become phosphorylated within 20 s of insulin binding (White et al., 1985, 1987, 1988; Kohanski et al., 1986) and produce a 20–200-fold stimulation in catalytic efficiency of the IR as a tyrosine kinase. This constellation of three tyrosyl residues in

Reprint requests to: Christopher T. Walsh, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, Massachusetts 02115.

Abbreviations: LAR, leukocyte antigen-related; PTPase, protein tyrosine phosphatase; IR, insulin receptor; pY, phosphotyrosine; IR peptide or YYY, TRDIYETDYYRK; pY1, monophosphotyrosyl IR peptide; pY2, diphosphotyrosyl IR peptide; pY3, triphosphotyrosyl IR peptide; YPP, TRDIYETDpYpYRK; PYP, TRDIpYETDYpYRK; PPY, TRDIpYETDpYYRK; pYn, multiply tyrosine-phosphorylated IR peptide; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; ROESY, rotating frame nuclear Overhauser spectroscopy; TSP, trimethyl silyl propionate; HPTPβ, human protein tryosine phosphatase β; MES, 2-[*N*-morpholino]ethane-sulfonic acid; TOCSY, total correlation spectroscopy; SCUBA, stimulated cross peaks under bleached alphas.

close proximity within the primary sequence is a feature of other transmembrane receptor tyrosine kinases in this class including the insulin-like growth factor receptor, the met and trk oncogenes, v-ros, and the DILR and sevenless enzyme in *Drosophila* (Hanks et al., 1988), and it is assumed that similar paradigms exist for autophosphorylation of each leading to turning on of tyrosine kinase activity. The tyrosine residue 1,150 of IR is homologous to tyrosine (416) in subdomain VII (Hanks et al., 1988) of src where again autophosphorylation upregulates src kinase activity.

Given that receptor tyrosine kinases turn themselves on by autophosphorylation, how do they get turned off and by what signalling mechanisms? It is not clear whether dephosphorylation controls the IR pathway, but much attention has recently been focused on transmembrane PTPases (Alexander, 1990; Saito & Streuli, 1991). The CD45 PTPase is a prototype of such a regulatory phosphatase and has been shown to play key roles in T-cell activation signal transduction by the src family kinase $p56^{lck}$ (Ostergaard et al., 1989; Ostergaard & Trowbridge, 1990; Trowbridge et al., 1991). However, CD45 is restricted to hematopoietic cell lineages (Trowbridge et al., 1991). In contrast, the related PTPase LAR is widely expressed, and the catalytic domain has been cloned, expressed in and purified from *Escherichia coli* (Streuli et al., 1989; Cho et al., 1991), and shown to have broad specificity on a range of specific phosphotyrosyl peptides (pY peptides) corresponding to known pY sites in proteins (Cho et al., 1991, 1992).

In this study we have utilized the catalytic domain of LAR PTPase to assess its ability to recognize and dephosphorylate a multiply phosphorylated site and have chosen as a model the triphosphotyrosyl site of the IR autophosphorylation domain with attention to regioselectivity at the three pY residues. To this end one needs both an analytical method and specific substrates. A recent publication by Levine et al. (1991) utilized a dodecapeptide encompassing residues 1,143–1,153 (and preceded by R instead of T at the N-terminus, which corresponds to position 1,142) of the insulin receptor *R*RDIYETDYYRK with a 48-kDa soluble catalytic autophosphorylation fragment of the insulin receptor to monitor regiospecificity and stoichiometry of phosphorylation by proton NMR. They could establish a highly ordered kinetic pattern of phosphorylation beginning at residue 9 (Y1,150), followed by residue 10 (1,151), and finally a trace amount at residue 5 (1,146). We have similarly used ^{1}H-NMR and the dodecapeptide TRDIYETDYYRK, except for the study of enzymic dephosphorylation, which has required the development of methods (Bannwarth & Kitas, 1992) for preparation in high stoichiometric yield and high regioisomeric purity of the seven phosphotyrosyl peptides required: the triphospho form (pY3), the three regioisomeric diphosphotyrosyl peptides (pY2), and the three monophosphotyrosyl species (pY1 forms) (Kitas et al., 1991). The availability of these multiply phosphorylated (pYn) dodecapeptides has now permitted the sequential assignment, kinetic deconvolution, and characterization of individual substrate properties with the LAR PTPase catalytic domain.

Results

Synthesis and characterization of dodecapeptides regiospecifically phosphorylated at multiple tyrosines

The IR autophosphorylation sites of consequence for kinase activity upregulation are tyrosines 1,146, 1,150, and 1,151. In the dodecapeptide TRDIYETDYYRK (1,142–1,153) these correspond respectively to residues 5, 9, and 10, and we will refer to them in this manner, e.g., Y5, Y9, and Y10. The triphospho form of the IR is maximally active as a tyrosine kinase for signal propagation so the fully phosphorylated pY3 form of the autophosphorylation dodecapeptide was tested as a PTPase substrate. Because the path a PTPase might catalyze from the pY3 to the pY2 and pY1 forms is not known and was to be analyzed, these six peptides were also prepared as described elsewhere (Bannwarth & Kitas, 1992) both by a global phosphorylation approach (Kitas et al., 1991) and by use of protected phosphotyrosine building blocks. The seven pY peptides used as PTPase substrates in Table 1 were each pure by HPLC analysis, gave the anticipated mass spectral analysis, and had phosphotyrosyl residues at the anticipated sites by amino acid sequence analysis (Bannwarth & Kitas, 1992). These synthetic approaches to multiply phosphorylated tyrosine peptides with precise site and stoichiometry control not only enabled the enzymatic studies described below but should also be of general value in deconvolution of related signal transduction pathways employing tyrosyl residue phosphorylation and dephosphorylation.

Steady-state kinetic properties of pY3, pY2, and pY1 forms of the IR dodecapeptide with the LAR PTPase catalytic domain

Each of the seven phosphotyrosine-containing peptides of Table 1 was tested as a substrate with the pure recombinant catalytic D1 fragment of human LAR (Streuli et al., 1989) for enzyme-catalyzed release of inorganic phosphate (Cho et al., 1991, 1992). All seven pY peptides were substrates and K_m and V_{max} values were determined and are presented in Table 1. We have previously reported the data for each of the pY1 regioisomers (Cho et al., 1991). The V_{max} values (33–96 μmol/min/mg) varied over a threefold range (Table 1), suggesting no impediment to recognition by the high negative charge density especially in the pY2 and pY3 species. The trend seen in the pY1 series (Cho et al., 1992), that phosphorylation at Y5 yields the lowest K_m value (Table 1), holds up in the pY2 series

Table 1. *LAR phosphatase kinetic constants for isomeric polyphosphoryltyrosyl peptides*[a]

	V_{max}	K_m	k_{cat}/K_m
PYY	96	27	240
YPY	33	280	7.8
YYP	45	710	4.2
PPY	52	35	100
YPP	61	302	13.5
PYP	45	28	108
PPP	72	45	107

[a] V_{max}, μmol/min/mg; K_m, μM; k_{cat}/K_m, 10^4 s^{-1} M^{-1}.

as well where a pY group at position 5 produces about a 10-fold increase in affinity as measured by K_m. pY9 is less good followed by pY10 in the pY1 series. The K_m and V_{max} data yield initial rates but reveal nothing about any regioselectivity of recognition. Thus, in the pY2 series PPY (i.e., pY5pY9Y10) and PYP (i.e., pY5Y9pY10) have K_m values of 35 μM and 28 μM, whereas YPP (i.e., Y5pY9pY10) has a K_m of 302 μM, suggesting the PO_3 group at Y5 will be removed selectively, although this is only inferred from such correlations. The YPP and PPP (pY5pY9pY10) data do suggest that the LAR PTPase is not affected significantly by electrostatic interaction of the neighboring pY9pY10 groups in steady-state catalysis.

To begin to answer whether the LAR catalytic domain did indeed show any regioselectivity with the triphospho IR dodecapeptide, we turned initially to product analysis by HPLC using the authentic pY2 and pY1 regioisomers as chromatographic standards. It quickly became apparent that only limited resolution of the manifold would be possible. For example, YYP and YPY were not readily resolvable and reproducibility was not ideal. It was possible to show qualitatively that PPY was converted mainly to YPY, and PYP mainly to YYP, by preferential dephosphorylation at Y5, but other isomers were less easily deconvoluted and the pattern of PPP dephosphorylation and the subsequent fate and/or accumulation of intermediates could not be determined. At this point we turned to NMR analysis.

^{1}H NMR assignment of IR-related peptides

A regiospecific NMR analysis of LAR tyrosine phosphatase activity relies on the ability to resolve and assign resonance lines, which uniquely correspond to each of the seven related pYn IR peptides and the fully dephosphorylated YYY peptide. Two-dimensional NMR analysis (Wüthrich, 1986) of YYY was used to determine scalar and dipolar connectivities necessary for the sequential assignment (Table 2). The optimal conditions for sequential assignment of this relatively small peptide are not the same as those for carrying out the phosphatase reaction. The greatest chemical shift dispersion for all possible tyrosyl or phosphotyrosyl ^{1}H-NMR resonances occurs near pH 6.0 (well within the pH optimum for this enzyme) (Cho et al., 1991). In contrast, to minimize exchange of amide protons with solvent, and thereby facilitate the NMR assignment, the pH should be between 3.0 and 4.0. In addition, one-dimensional spectral quality was noticeably improved at 25 °C, whereas at 4 °C the number of cross peaks found within the ROESY spectrum was substantially improved due to an increase in rotational correlation time. Therefore, the assignment was carried out at pH 4.0 and 10 °C, whereas the enzymatic reactions were monitored by NMR at pH 6.0 and 25 °C. This required an extrapolation of the chemical shifts for amide,

Table 2. *Resonance assignments of TRDIYETDYYRK at 10 °C and pH 4.0*

		Chemical shift (ppm) with respect to TSP						
		Amide	α	β	γ	δ	(2,6)	(3,5)
1	Thr		4.16	3.88	1.31			
2	Arg	8.91	4.35	1.79 (1.77)	1.60	2.98		
3	Asp	8.70	4.66	2.80 (2.73)				
4	Ile	8.20	4.04	1.75	1.07 (1.00) 0.74 (methyl)	0.73		
5	Tyr	8.17	4.57	3.09 (2.90)			7.09	6.80
6	Glu	8.14	4.38	2.10 (1.96)	2.39			
7	Thr	8.26	4.22	4.14	1.15			
8	Asp	8.49	4.64	2.79 (2.75)				
9	Tyr	8.10	4.40	2.88			6.91	6.76
10	Tyr	8.00	4.44	3.01 (2.91)			7.10	6.82
11	Arg	8.01	4.22	1.80	1.67 (1.55)	3.16		
12	Lys	8.15	4.17	1.85 (1.76)	1.40	1.82 4.14 (ε)		

Table 3. *Chemical shift changes for the aromatic resonances of the tyrosyl residues extrapolated between the above conditions and 25 °C and pH 6*

		Y5		Y9		Y10	
pH	°C	(2,6)	(3,5)	(2,6)	(3,5)	(2,6)	(3,5)
6.0	25	7.133	6.805	6.906	6.773	7.009	6.855
5.5	25	7.132	6.803	6.906	6.771	7.101	6.856
5.0	25	7.124	6.801	6.910	6.768	7.106	6.846
4.5	25	7.113	6.798	6.918	6.767	7.102	6.838
4.0	25	7.104	6.798	6.927	6.769	7.101	6.832
4.0	20	7.105	6.796	6.923	6.766	7.101	6.831
4.0	15	7.105	6.794	6.917	6.762	7.102	6.830
4.0	10	7.094	6.796	6.911	6.756	7.099	6.828

arginine side chain, and tyrosyl ring protons through changes of pH (from 4.0 to 6.0) and temperature (from 10 to 25 °C) (Table 3). Using these modifications of sample conditions, we were able to resolve each spin system uniquely and obtain at least two interresidue connectivities for each residue-to-residue step in the sequence. The results are summarized in Table 2. The absolute shift of the tyrosyl protons in YYY is +0.03 ppm downfield from an independent determination in the previously reported study at pH 7.5 (Levine et al., 1991), whereas the characteristic pattern is identical. The pattern was compared with that recorded for the synthetic regioisomeric pY1 peptides (compare Fig. 1, YYY, with the three pY1 regioisomers, PYY, YPY, and YYP). YPY and PYY demonstrate only localized perturbations of the ring proton chemical shifts, allowing for the direct assignment of the tyrosyl resonances. In contrast, the addition of a phosphoryl group to Tyr 10 (YYP) results in more sizable perturbations to the chemical shifts for the protons of all three of the tyrosyl groups. Potential ambiguities between Tyr 5 and Tyr 9 of YYP were resolved by comparison of the spectra for YYP with PYP in Figure 1, as well as selective proton homonuclear decoupling experiments (data not shown). Assignments of the resonances for all of the tyrosyl residues in the three regioisomeric diphosphotyrosyl peptides were performed similarly. Through the use of this methodology we developed specific quantifiable marker resonances for each of the seven pYn peptides and the fully dephosphorylated peptide. All of these markers are differentiable based upon a unique pattern of resonances between 6.70 and 7.03 ppm. Only the PPP sample had no signals in this "fingerprint region." These markers make it possible to characterize the various pools of regioisomeric phosphorylated peptides at any point along the dephosphorylation reaction manifold from PPP to YYY. The assignments for phosphotyrosyl residues within the pYn-containing peptides are given in Table 4. Because of near degeneracy for many of the resonances in the pY residues, our assignments were confined to identifying unique marker resonances for each of the pYn-containing peptides.

LAR dephosphorylation of PPP monitored by ^{1}H-NMR

The initial reaction is the conversion of PPP to YPP

To extend our study beyond the limitations encountered with HPLC analysis we incubated PPP with LAR PTPase and followed the reaction using ^{1}H-NMR to monitor the marker resonances for each of the possible pY2 and pY1 intermediates formed via dephosphorylation (Figs. 2, 3). Initially, a resonance at 7.21 ppm (peak **A** of Fig. 2) disappears rapidly while a new resonance simultaneously begins to appear at 6.82 ppm (peak **B** of Fig. 2). A comparison of the intensities of these two resonances as a function of time is shown in series **a** and **b** of Figure 3. Peak **A** is undetectable by 41 min, and in a reciprocal fashion, peak **B** simultaneously reaches its maximum by this time point and subsequently decreases below detection by 100 min. This precursor-product rela-

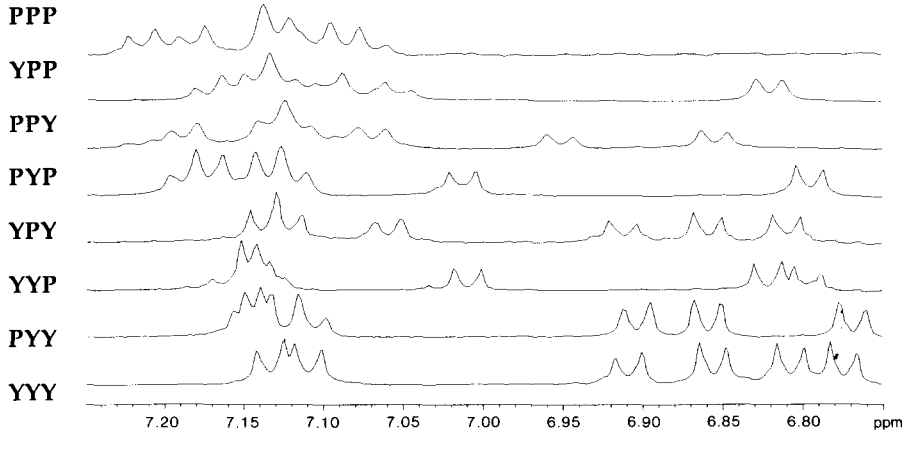

Fig. 1. ^{1}H-NMR spectra of the aromatic region for YYY and the pYn regioisomeric IR peptides. Spectra were recorded at pH 6.0 and 25 °C; peptide concentrations were between 0.6 and 2.9 mM.

Table 4. *Chemical shift assignments for the aromatic resonances of the phosphotyrosyl residues found in each of the seven pYn peptides*

	Y5		Y9		Y10	
	(2,6)	(3,5)	(2,6)	(3,5)	(2,6)	(3,5)
PPP	*[a]	*	*	*	*	*
YPP	*	6.820	*	*	*	*
PPY	*	*	*	*	6.952	6.855
PYP	*	*	7.010	6.797	*	*
YPY	7.148	6.811	6.911	7.058	7.120	6.855
YYP	7.010	6.825	6.799	*	*	*
PYY	7.149	7.140	6.902	6.770	7.005	6.859

[a] Asterisk indicates not determined due to overlap.

tionship is almost exclusively confined to these two signals (a minor side reaction is discussed below). By comparing the spectra for the pY2 regioisomers in Figure 1, the patterns are found to be quite unique. In particular the pattern for YPP, with a lone fingerprint resonance at 6.82 ppm, is identical with that of the predominant product formed during the initial stages of dephosphorylation of PPP. Because the LAR PTPase-dependent dephosphorylation of PPP to YPP proceeds almost exclusively with respect to the other two potential reactions (i.e., to PYP or PPY), it is concluded that the initial step within this pathway occurs with a high degree of regiospecificity. This result allows the remainder of the dephosphorylation pathway to be substantially restricted in subsequent studies. Moreover, it thus follows that there should be little pY2 or pY1 substrate available with a phosphotyrosyl residue at position 5. For example, in the pY2 series the peptides PYP and PPY can be thought to represent minor substrate pools for the next step of the LAR PTPase activity. We therefore can confine our attention to the other four peptides (of Table 1), dramatically simplifying the complexity of the study. Although the accumulation of at least one intermediate pool along the pathway could not be clearly predicted a priori, the data clearly demonstrate that the relative regioselectivity observed is sufficient to allow for the observation of discrete and transient phosphorylation state(s) within the reaction manifold.

Minor amounts of PPY are also formed initially

During the initial 41 min, a second peak also begins to appear at 6.93 ppm (peak **C** of Fig. 2). However, this peak is perceptibly slower in accumulation than peak **B**, and never approaches equivalent magnitude (series **c** of Fig. 3). Furthermore peak **C**, which is the unique marker for PPY, becomes undetectable after 41 min. The relative amount of PPY to YPP can be roughly quantitated through signal integration and has been estimated to be less than 10% (data not shown).

YYP accumulates as an intermediate

A third marker resonance at 7.01 ppm (peak **D** of Fig. 2) has begun to appear by 26 min, and then modestly continues until reaching a maximum at 46 min and then decays by 61 min. Peak **D** cannot be unambiguously assigned to either the pY2 species PYP or the pY1 species YYP because of signal overlap. The signals that differentiate these two marker resonances are beneath more intense signals that are located elsewhere within the spectra. However, as noted above, it can be argued that a significant pool of PYP is inconsistent with the data of Figures 2 and 3. Therefore the accumulation of a YYP pool follows directly. This secondary precursor-product relationship results from the dephosphorylation of the nascent YPP. Series **d** in Figure 3 is the timecourse of this pool. The accumulation of YYP follows the rapid initial flux of PPP to YPP described above and precedes the final product accumulation depicted in the bottom series of Figure 3. Flux through this pool thus defines a second discrete transient phosphorylation state within the reaction manifold.

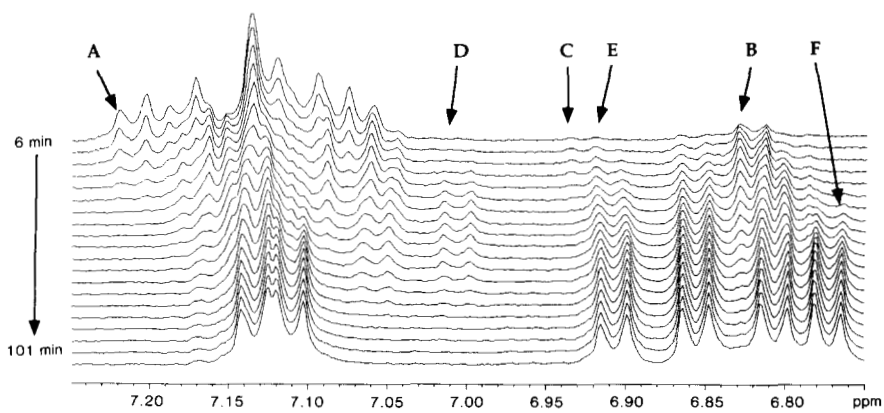

Fig. 2. ^{1}H-NMR of the aromatic region for the IR peptides monitoring the reaction of PPP (600 μM) with LAR PTPase (46.3 ng/mL) at pH 6.0 and 25 °C. From top to bottom are 5-min averages starting at 3.5 min after addition of the enzyme, i.e., time points are averages at 6, 11, 16, 21, 26, 31, 36, 42, 46, 51, 56, 61, 66, 71, 76, 81, 86, 91, 96, and 101 min. Peak **A** at 7.21 ppm represents the disappearance of PPP, peak **B** (6.82 ppm) represents the transient appearance of YPP, peak **C** (6.93 ppm) represents PPY, peak **D** (7.01 ppm) represents YYP, peak **E** (6.91 ppm) represents both YYY and YPY, and peak **F** (6.77 ppm) is only from the final product YYY.

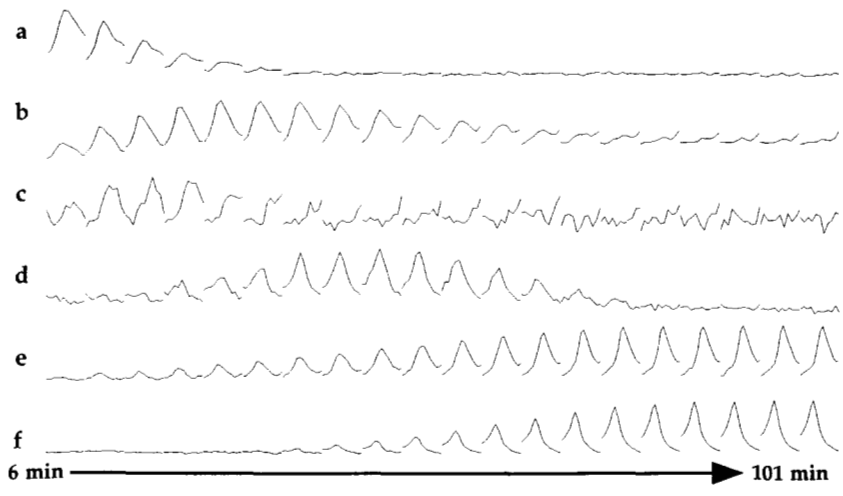

Fig. 3. Individual normalized peak intensities from Figure 2 are depicted as a function of time (left to right corresponds to the average beginning at 6 min and continuing to 101 min). Proceeding from top to bottom, kinetic series **a** through **f** correspond to Figure 2 peaks **A** through **F**.

YPY also accumulates prior to complete formation of YYY

The predominant reaction products from the incubation of the LAR PTPase soluble domain with PPP have begun to accumulate by 40 min (e.g., at 6.91 and 6.77 ppm, peaks **E** and **F**, respectively, of Fig. 2) (as well as peaks at 6.81, 6.86, 7.11, and 7.33 ppm). Collectively, these peaks are the marker resonances for YYY, YPY, or PYY. Through analogy to earlier arguments, PYY is excluded from this group of potential marker resonances. Although many of the signals for YYY and YPY are degenerate, resonances **E** and **F** allow these two species to be distinguished. Resonance **F** corresponds exclusively to YYY, the product of complete dephosphorylation of PPP. However, resonance **E** is potentially a composite signal of two nearly degenerate resonances that are markers for both YPY and YYY. Transient accumulation of YPY is evidenced within peak **E** in two ways. First, the chemical shift of peak **E** is observed to undergo an apparent small shift of approximately −0.003 ppm throughout the reaction as depicted in Figure 2. Note that the fingerprints of YPY and YYY differ by approximately the same amount (Table 3), and other resonances within Figure 2 do not demonstrate similar behavior during the course of the reaction. Second, the intensity change for resonance **E** is more intense than that for resonance **F** during the period from 21 to 61 min, demonstrating that these two peaks cannot represent the appearance of a single species. The change in chemical shift as well as the difference in the rate of increase for these two resonances is interpreted as a measure of the transient accumulation of YPY (Fig. 3, series **e** and **f**). The maximum difference in signal intensity is at approximately the same time point as that for the maximum accumulation of YYP (series **d** of Fig. 3). Comparison of the data for the deconvolution of the pY2 series with data for the pY1 series (i.e., observation of only YPP compared with observation of both YPY and YYP) indicates that there is an apparent decrease in the relative regioselectivity of dephosphorylation.

Dephosphorylation of PYP, PPY, and YPP with LAR PTPase

The three regioisomeric pY2 peptides were also separately incubated as substrates for the LAR PTPase catalytic domain. The results for the two peptides with a phosphoryl group on Tyr5 (PYP and PPY) are nearly identical. The regioselectivity observed for the first reaction with PPP was also observed for these two peptides. Essentially complete loss of the first phosphate from position 5 occurs relatively quickly prior to significant dephosphorylation at either position 9 or 10 (data not shown). Although evidence does exist that minor amounts of these two peptides can be found on the pathway from PPP to YYY, it is clear that they are efficiently converted to pY1 regioisomers via preferential removal of the phosphate group from the tyrosyl residue at position 5. These results present an additional argument against the observance of significant amounts of PYY within the reaction manifold. The high degree of regiospecificity observed for pY5 is thus borne out at multiple stages of the pathway. Similarly, the results from the determination of V_{max} and K_m for pY1 peptides (Table 1) have further demonstrated this regiospecificity. Thus, the high degree of regiospecificity for the predominant dephosphorylation at Tyr5 is continually maintained as a simplifying constrictive pressure across the entire manifold at the pY3, pY2, and pY1 stages.

The NMR analysis of dephosphorylation of YPP was of particular interest in determining whether the differences measured for the kinetic constants could be correlated with an observable regioselectivity. The conversion of this pY2 species to one of two possible pY1 regioisomers proceeded with a negligible preference for dephosphorylation at either position 9 or 10 (Fig. 4). However,

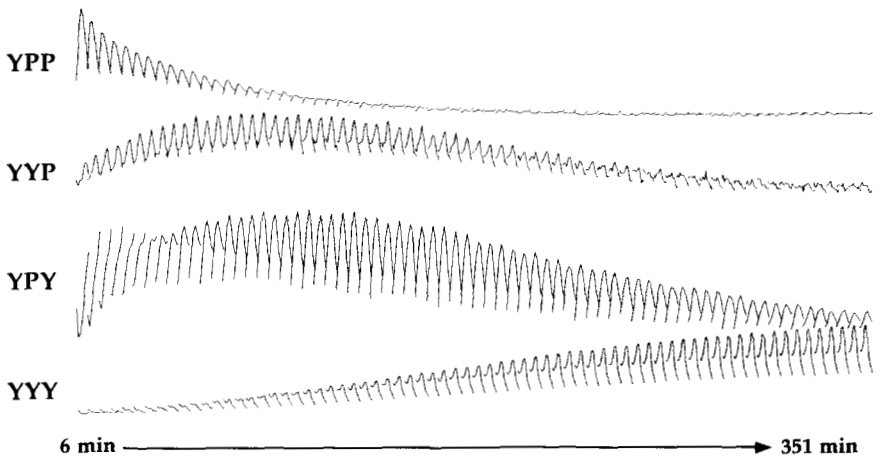

Fig. 4. Analogous to Figure 3, these normalized kinetic series are from 6 to 351 min (5 min each). From top to bottom, the disappearance of YPP (**B**), transient appearance of YYP (**D**) and YPY (peak at 7.04–7.05 ppm), and formation of YYY (**F**).

although neither site was of significant preference in the pY2 to pY1 reaction (appearance of YPY and YYP in Fig. 4), it is obvious that disappearance of the pY2 species (disappearance of YPP, Fig. 4) is nearly complete prior to significant accumulation of the completely dephosphorylated peptide (accumulation of the product YYY, Fig. 4), demonstrating a preference for the pY2 to pY1 reaction over that for pY1 to YYY.

Discussion

The development of synthetic methods for regioselective phosphorylation of multiple tyrosyl residues in unblocked peptides in high yields and purities (Bannwarth & Kitas, 1992) has permitted the accumulation of samples of the dodecapeptide-encompassing residues 1,142–1,153 of the key autophosphorylation site of the tyrosine kinase domain of the insulin receptor. Sufficient quantities were prepared for distinction of the pY3 form, the three pY2 isomers, and the three pY1 isomers from the Y3 form by analysis of the aromatic region of ^{1}H-NMR spectra. These data corroborate and extend NMR assignments made recently by Levine et al. (1991) in their study of patterns of phosphorylation of the Y3 form by a soluble catalytic fragment of IR. We have utilized these eight peptides to probe the specificity of the soluble 40-kDa catalytic domain of a widely expressed transmembrane receptor PTPase, LAR, for action at multiple phosphotyrosyl residues. Although it is yet unclear whether LAR and/or some other PTPases, separately or interactively, have a functional role in vivo for dephosphorylation and deactivation of the kinase activity of the insulin receptor, this study is prototypic for pYn regioisomers and presents a general method for analysis of precursor–intermediate–product relationships in tyrosine-specific phosphatase action at multiply phosphorylated sites.

LAR shows high specificity in initiating dephosphorylation of a pY residue at position 5 (1,146 in IR) in the dodecapeptide over residues 9 and 10 at all phosphorylation levels, triphosphotyrosyl, diphosphotyrosyl, and monophosphotyrosyl. Thus >90% of the flux of the pY3 substrate is to the YPP form of the pY2 product with small amounts of the other pY2 species detectable. As noted from Figure 3, the YPP diphosphotyrosyl regioisomer builds up to a level that is then processed by the PTPase to monophosphotyrosyl product with little preference for dephosphorylation at either position 9 or 10 to yield comparable amounts of both YPY and YYP. Both monophosphotyrosyl isomers are subsequently dephosphorylated to the final product unphosphorylated YYY as schematized in Figure 5. The relatively small amount formed of the pY2 species PPY is preferentially dephosphorylated at position 5 to also yield YPY in the reaction manifold. Rare species in the reaction manifold from NMR analysis are PYP at the pY2 level and PYY at the pY1 level. Most of the reaction flux in the sequential conversion of triphospho to diphospho forms of the IR autophosphorylation peptide is thus through loss of the

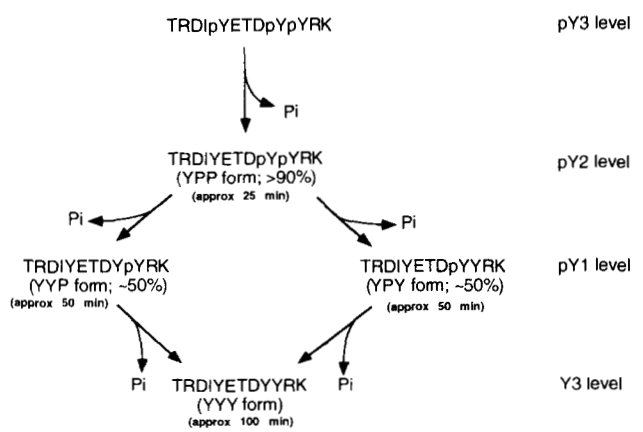

Fig. 5. Schematized regioselectivity of action of LAR PPTPase catalytic fragment on the autophosphorylation site dodecapeptide of the activated insulin receptor kinase domain. The majority of flux to pY2 and pY1 species is indicated. See text for a discussion of minor pY2 forms.

5 phosphoryl group, followed by loss of either the 9 or 10 phosphoryl group and then dephosphorylation of the pY1 species to YYY. These results are in agreement with a similar study (Ramachandran et al., 1992), which appeared during the preparation of this manuscript, where solid-phase sequencing was used to monitor sequential dephosphorylation of the IR peptide.

The recent study by Levine et al. (1991) in the opposite direction from Y3 to pY3 with the IR catalytic fragment as tyrosine kinase catalyst yielded the opposite regiospecificity, initial phosphorylation at Y9, then at Y10, and only very slowly at Y5. In this regard with this dodecapeptide substrate the last $-PO_3$ added (at position 5 by the IR PTK) is the first removed (by LAR PTPase), the second $-PO_3$ added at position 10 is removed relatively slowly, whereas a $-PO_3$ is preferentially added to position 9 by the kinase and yet is removed relatively slowly by the phosphatase. However, one should not rush from in vitro specificities for an autophosphorylation peptide to clear implications of in vivo specificity without cautionary flags. Studies on the autophosphorylation of full-length IR suggest that the pY2 species, rapidly accumulating from the Y3 species upon insulin binding, corresponds to the PYP and PPY peptides used in this study. The predominance of a phosphotyrosyl group at position 5 (White et al., 1988) is in contrast to the in vitro results with the IR catalytic fragment. On the other hand, recent mutagenesis studies indicate a Y1146F mutant can still be fully activated for IR kinase activity in COS cell transfections (Murakami & Rosen, 1991), whereas Y1,150 and Y1,151 are crucial, suggesting that the pY1,150 and pY1,151 forms are essential in autoactivation of IR tyrosine kinase activity. One could perhaps test LAR PTPase with full-length IR β subunits for dephosphorylation regiospecificity but NMR analysis may become more problematic with such a large molecular weight polypeptide, and it is unclear how to get the high concentrations of pY3 and pY2 full-length species needed for NMR.

The triphosphotyrosyl IR dodecapeptide may be a useful model substrate to probe specificity of recognition by other receptor transmembrane PTPases, e.g., human HPTPβ, which only has a single, rather than a tandemly repeated 300-residue PTPase domain (Krueger et al., 1990), and also by soluble PTPases. Such studies might suggest which PTPase genes would be good candidates for knockouts in transgenic studies to assess in vivo deactivation routes for triphospho IR. The spacing of three invariant tyrosyl groups in seven residues is conserved in several members of the IR tyrosine kinase family with some sequence variation in the interposed four residues in the met and trk oncogenes (Hanks et al., 1988). These may undergo autophosphorylative kinase autoactivation in signal transduction; specificity for distinct PTPase action could be tested at the level of pY3 forms of the variant dodecapeptide sequences. Finally, an ability to extend regio- and chemoselective side-chain phosphorylations to synthesize both phosphothreonyl and phosphotyrosyl residues in small peptides would permit one to use this NMR approach to ask such questions as whether the cdc25 phosphatase (Gautier et al., 1991; Kumagai & Dunphy, 1991) dephosphorylates both the pT and pY residues in the pTEpY sequence of the ATP binding site of the cell cycle kinase p34cdc2 (Gould & Nurse, 1989; Solomon et al., 1990; Krek & Nigg, 1991) and to determine whether there is a kinetic order for dephosphorylation in initiation of mitosis.

Materials and methods

Phosphopeptides

The dodecapeptides used in these studies include the unphosphorylated TRDIYETDYYRK form (residues 1,142–1,153 of the IR, termed the YYY form) and the three pY1 forms—TRDIpYETDYYRK (PYY form), TRDIYETD pYYRK (YPY form), and TRDIYETDYpYRK (YYP form)—reported earlier (Kitas et al., 1991; Cho et al., 1992). The three pY2 forms—TRDIpYETDpYYRK (PPY form), TRDIpYETDYpYRK (PYP form), TRDI YETDpYpYRK (YPP form)—and the pY3 form TRDI pYETDpYpYRK (PPP form) were prepared as reported in Bannwarth and Kitas (1992).

Preparation of the phosphatase

The 40-kDa catalytic fragment corresponding to residues 1,275–1,613 of human LAR PTPase was purified to homogeneity from an *E. coli* expression system as previously reported (Cho et al., 1991).

Steady-state kinetic assay

Steady-state kinetic parameters were determined as described previously (Cho et al., 1991, 1992). Briefly, the release of inorganic phosphate by enzymatic hydrolysis of phosphotyrosyl peptides was determined by malachite green color reagent (Lanzetta et al., 1979; Martin et al., 1985). LAR was diluted with LAR buffer (25 mM HEPES, 2 mM EDTA, 10 mM DTT, 1.0 mg/mL bovine serum albumin, pH 7.3) and added to the reaction mixture containing the phosphopeptide LAR reaction buffer (100 mM MES, 2 mM EDTA, 10 mM DTT, pH 6.0). Typically the total reaction volume was 50–500 μL after the addition of 5 or 10 μL (20–300 ng) of diluted enzyme, and the reaction mixture was incubated at 25 °C for 2–4 min. The assay was quenched with 900 μL of the malachite green color reagent, and after 10 min at room temperature, the absorbance at 660 nm was determined. The quantity of Pi was calculated by comparison to a standard curve determined for inorganic phosphate.

NMR experiments

For assignment purposes, samples of IR peptide were dissolved in 50 mM d4-acetic acid in either 10% or "100%" D_2O, and the pH was adjusted with NaOD. The pH values were direct meter readings. ROESY experiments were performed by the method of Griesinger and Ernst (1987), using a CW field of 4.7 kHz for 200 ms. TOCSY experiments (Braunschweiler & Ernst, 1983) were performed using a 55-ms DIPSI-3 sequence (Shaka et al., 1988) for the spin lock at a field strength of 10 kHz. DQf-COSY was performed according to Muller et al. (1985). Water suppression was performed using transmitter presaturation followed by a SCUBA sequence (Brown et al., 1988). Steady-state was achieved using two orthogonal pulses at the beginning of each transient, which were adjusted to at least 15 times the 90° pulse width.

LAR phosphatase reaction samples were prepared by dissolving regioisomeric phosphotyrosyl peptides in 450 μL of 50 mM d_4-acetic acid in 99.9% D_2O containing 100 μg/mL BSA. The pH was adjusted to 6.0 with NaOD and the volume adjusted to 500 μL. An aliquot of 10 μL was removed for phosphate assay. Typical concentrations of phosphopeptides were 0.6–1.7 mM. The samples were then used to setup and record a $t = 0$ spectrum for each sample. Fifty microliters of LAR PTPase (500 ng/mL), in the reaction buffer, was then added (final concentration 46.3 ng/mL), and data acquisition commenced within 3–4 min. One second of transmitter presaturation was followed by a 60° pulse. The total cycle time was 2.34 s for acquisition of 16,000 complex data points. Each kinetic data point was the result of 128 averages during 5 min. Sample temperature was maintained at 25 °C.

The spectra were acquired on a Varian UNITY 500S NMR spectrometer operating at 499.843 MHz. All data were acquired and processed using the VNMRS 4.1 beta software package from Varian Associates and processed on SUN microsystems workstations.

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