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Synergistic activities of multiple phosphotyrosine residues mediate full signaling from the *Drosophila* Torso receptor tyrosine kinase

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ABSTRACT **Here, we identify four tyrosine residues (Y644, Y698, Y767, and Y772) that become phosphorylated after activation of the Torso (Tor) receptor tyrosine kinase. Previously, we characterized phosphotyrosine sites (P-Y630 and P-Y918). Of the six P-Y sites identified, three (Y630, Y644, and Y698) are located in the kinase domain insert region, one (Y918) is located in the C-terminal tail region, and two (Y767 and Y772) are located in the activation loop of the kinase domain. To investigate the function of each P-Y residue in Tor signaling, we have generated transgenic Drosophila embryos expressing mutant Tor receptors containing either single or multiple tyrosine to phenylalanine substitutions. Single P-Y mutations were found to have either positive, negative, or no effect on the signaling activity of the receptor. Elimination of all P-Y sites within the kinase insert region resulted in the complete loss of receptor function, indicating that some combination of these sites is necessary for Tor signaling. Mutation of the C-terminal P-Y918 site revealed that this site is responsible for negative signaling or down-regulation of receptor activity. Mutation of the P-Y sites in the kinase domain activation loop demonstrated that these sites are essential for enzymatic activity. Our analysis provides a detailed *in vivo* example of the extent of cooperativity between P-Y residues in transducing the signal received by a receptor tyrosine kinase and *in vivo* data demonstrating the function of P-Y residues in the activation loop of the kinase domain.**

Receptor tyrosine kinases (RTKs) function in developmental, mitogenic, and oncogenic signal transduction pathways in organisms as diverse as mammals, *Drosophila melanogaster* and *Caenorhabditis elegans* (1–3). After their activation by extracellular ligands, these receptors dimerize and become phosphorylated on specific tyrosine residues in the intracellular domain (4). The phosphorylated tyrosine (P-Y) residues function as docking sites for a number of cytosolic molecules such as Src homology 2 (SH2) domain-containing proteins (5, 6) and P-Y binding (PTB) domain-containing proteins (7, 8). In the past few years, extensive progress has been made in understanding how the signal is transmitted from the receptor to the nucleus. However, the complete picture of how specific RTKs regulate the activity of downstream molecules to transduce a signal *in vivo* is still not clear. For example, extensive studies of the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors have identified the sites of tyrosine phosphorylation on these RTKs and the signaling molecules that are able to interact with these sites. However, analyses in cell culture assays of the function of these sites, and thus of the binding proteins, have not always provided a clear answer of their contributions to the signaling output (9, 10).

To substantiate findings obtained from tissue culture systems, it is important to conduct similar analyses in a true *in vivo* setting. To this end, model systems such as *Drosophila* and *C. elegans* provide useful assays. In *Drosophila*, the Torso (Tor) receptor is an excellent system to dissect both the *in vivo* and *in vitro* signaling events regulated by a RTK. In the developing Drosophila embryo, localized activation of Tor at the embryonic termini leads to formation of specialized anterior and posterior terminal structures, termed acron and telson, respectively (for review, see refs. 11 and 12). After activation, Tor molecules become phosphorylated and activate a signaling pathway that includes the nonreceptor tyrosine phosphatase Corkscrew (Csw; a.k.a. SHP-2), the adapter molecules Drk and Daughter of Sevenless, and the small GTPase Ras1 ($p21^{Ras}$) and its regulators: the exchange factor Son of Sevenless and Gap1; the 14-3-3 protein Leonardo; and the Raf, MEK, KSR, and Rolled kinases (13–16). The output of Tor signaling can be visualized at the level of expression of the transcription factors *tailless* (*tll*) and *huckebein* (*hkb*) as well as at the level of the embryonic cuticle. Posteriorly, the domain of expression of *tll* and *hkb* transcription factors provide a "read out" of the level of Tor activity (13, 14, 17, 18).

To characterize the mechanism by which Tor regulates this complex signaling cascade, we have undertaken a structure/function analysis of Tor. Previously, we identified two major P-Y sites that become phosphorylated after Tor activation (19). The first site, P-Y630, serves to positively transduce the Tor signal and mediates binding to the Csw protein. Mutation of P-Y630 reduces Tor activity, which is consistent with the phenotype of *csw* mutants (20). The second site, P-Y918, mediates binding to Drosophila RasGAP (21) and is involved in negative signaling because deletion of this site *in vivo* is associated with a Tor gof phenotype. The fact that residual activity still could be detected in tor^{Y630F} and $tor^{YY630+918FF}$ mutants suggests that Tor regulates downstream events through either another P-Y site(s) on the receptor or, as previously proposed for Sev (22), through a P-Y-independent mechanism. To address this question, we have expanded our structure/function analysis of the Tor RTK. Further mapping of P-Y residues revealed the presence of at least four additional sites of phosphorylation, Y644, Y698, Y767, and Y772. Using a series of mutant receptors containing single or multiple tyrosine to phenylalanine substitutions, we have characterized the *in vivo* function of each of these sites and have found that mutating all of the tyrosine residues in the kinase insert region results in a complete Tor lof phenotype. Our analysis demonstrates that Tor does not signal when a combination of either two or three P-Y residues in the kinase insert domain are mutated, thus illustrating the cooperativity between P-Y residues in transducing the Tor signal. Finally, we find that both P-Y sites in the kinase domain activation loop (Y767 and Y772) are

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Abbreviations: RTK, receptor tyrosine kinase; Tor, Torso; SH2, Src homology 2; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; Csw, Corkscrew phosphatase; *tll*, *tailless* transcription factor; *hkb*, *huckebein* transcription factor; WT, wild-type.

necessary for Tor catalytic activity and that mutation of these sites leads to a complete Tor lof phenotype.

MATERIALS AND METHODS

Antibodies. The Tor antibody used in this analysis was a polyclonal rabbit antibody directed against the cytoplasmic domain of Tor (23). The phosphotyrosine antibody used was 4G10 (Upstate Biotechnology).

Construction of Vectors Encoding Mutant Tor Proteins. For phosphorylation site mapping analysis, a cDNA clone encoding wild-type (WT) Tor (pBtor; ref. 23) was digested with *Bam*HI and *Eco*RI, and the fragment containing the entire *tor* coding sequences was inserted into the *Nco*I-*Eco*RI cloning sites of the pGem-7Zf plasmid (Promega). *tor* constructs encoding amino acid point mutations were generated by site-directed mutagenesis as described (24) using pGemTor and the appropriate oligonucleotides to introduce the desired base changes. cDNA fragments encoding the entire WT and mutant Tor proteins were isolated and inserted into the pAcC4 baculoviral transfer vector for expression in Sf9 cells.

For the generation of transgenic embryos, the 11.5-kilobase genomic *tor* rescue fragment was digested with *Sna*B1 and *Nhe*I and was ligated into the Litmus39 (NEB, Beverly, MA) vector (see ref. 19). Point mutations were generated by site-directed mutagenesis as described (24). The specific base changes in all mutant constructs were confirmed by sequence analysis. After mutagenesis, the constructs were ligated into the 11.5-kilobase genomic background and were used for injection into *Drosophila* embryos following standard procedures (see below).

Expression of Recombinant Tor Proteins, Preparation of Cellular Lysates, and Immunoprecipitation Assays. Recombinant Tor proteins were expressed in Sf9 cells by using the appropriate baculovirus vector. The Sf9 cells then were washed twice with ice cold PBS and were lysed for 20 minutes at 4°C in 500 μl of Nonidet-40 (Nonidet P-40) lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonylflouride, 0.15 units/ml aprotinin, 20 μM leupeptin, and 5 mM sodium vanadate. Insoluble material was removed by centrifugation at 4°C for 10 min at 10,000 $\times$ *g*, and cell lysates were equalized for Tor protein expression by immunoblot analysis. For *Drosophila* embryo extracts, 0.05 mg of embryos were resuspended in 1 ml of Nonidet P-40 lysis buffer. Embryos then were dounced six strokes in a ground–glass dounce (Wheaton Scientific) and were incubated on ice for 15 min. Insoluble material was removed from the lysates as described above. Immunoprecipitation assays were performed by incubating lysates with the appropriate antibody for 3 h at 4°C. Protein A/G agarose beads (Santa Cruz Biotechnology) were used to collect the antigen–antibody complexes. The immunoprecipitates then were washed four times with cold lysis buffer before analysis.

***In Vitro* Kinase Assays and Phosphorylation Site Mapping.** Tor immunoprecipitates were washed three times with Nonidet P-40 lysis buffer containing 1 mM sodium vanadate and once with kinase buffer (30 mM Hepes, pH 7.4/10 mM $MnCl_2$/5 mM $MgCl_2$/1 mM DTT/5 μM ATP). The complexes then were incubated at 25°C for 15 min in 40 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP. Kinase assays were terminated by the addition of gel loading buffer (4% SDS/80 mM DTT/10% glycerol), the samples were resolved by SDS/PAGE), and the phosphoproteins were visualized by autoradiography. Phosphoproteins then were analyzed by reversed-phase HPLC, phosphoamino acid analysis, and Edman degradation as described (19).

Generation of Transgenic Flies. An 11.5-kilobase *Eco*RI-*Eco*RI genomic *tor* DNA fragment previously has been shown to fully rescue the *tor* null phenotype (25) and was used to test the activity of various *tor* mutations (see also ref. 19). Constructs encoding mutant Tor proteins (Y644F, Y656F, Y698F, YY767 + 772FF, quadruple, quintet, or δ insert) were generated into CasPeR4 (26), which contains the marker *mini-white*. P-element-mediated transformation was performed according to Spradling (27) by coinjecting with the helper plasmid pUCSHpdelta2–3 at a concentration of 0.5 mg/ml (28). Transformant lines with insertions on the X and third chromosome were used to generate flies that carry one or two copies of *P[tor$^+$]* in a *tor*^{XR1} background. *tor*^{XR1} contains a deletion within the *tor* gene that does not make a protein (23, 25).

Examination of Embryos. Embryos 0–3 h old were collected and prepared for *in situ* hybridization by using digoxygenin-labeled probes as described by Tautz and Pfeifle (29). Single-stranded sense and antisense digoxigenin containing DNA probes were prepared by the PCR labeling technique by using appropriate primers (Biolabs, Northbrook, IL). Probes were prepared from plasmids containing the *tll* (30) and *hkb* (31) cDNAs. For visualization, embryos were mounted in glycerol. Subsequently embryos were analyzed and photographed with a Zeiss Axiophot microscope equipped with Nomarski optics. Embryonic cuticles were prepared in Hoyer's mountant as described by van der Meer (32) from 24- to 36-h-old embryos.

RESULTS

Identification of Phosphotyrosine Sites on Tor. Previously, we identified Y630 and Y918 as the major sites of Tor autophosphorylation (19). However, mutation of these sites (YY630 + 918FF) does not completely eliminate Tor autophosphorylation because additional tyrosine-phosphorylated tryptic peptides can be observed in both YY630 + 918FF Tor and WT Tor proteins. In addition, mutation of both P-Y sites does not eliminate the *in vivo* Tor signal. To identify these additional sites, activated Tor proteins were immunoprecipitated, were washed extensively, and were allowed to autophosphorylate in the presence of Mn^{+2} and [γ-^{32}P]ATP. The ^{32}P-labeled Tor protein was electrophoresed on SDS-polyacrylamide gels, was isolated from the gel matrix, and was digested with trypsin. The tryptic phosphopeptides then were separated and eluted from a reversed-phase HPLC C-18 column. When the radioactivity released from the column was analyzed, two major peaks (peaks 1 and 4) and three smaller peaks (peaks 2, 3, and 5) were detected (Fig. 1*A*). Phosphoamino acid analysis of the peptides contained in these peaks indicates that they are phosphorylated exclusively on tyrosine. We previously have determined (19) that peaks 1 and 4 represent P-Y630 and 918, respectively. To identify the site(s) phosphorylated in the remaining peaks (peak 2, 3, and 5), a Tor$^{Delta\text{-}Insert}$ protein that lacks the kinase domain insert region was analyzed as described above. As shown in Fig. 1*B*, peak 4s and 1a, a new peak that previously had been undetected because of comigration with peak 1, were observed. Because of the disappearance of peaks 1, 2, 3, and 5 from the Tor$^{Delta\text{-}Insert}$ protein, we conclude that these peaks contain P-Y residues located in the Tor insert region whereas the P-Y residues contained in peaks 1a and 4 are located outside this region.

Identification of Phosphotyrosine Sites Y767 and Y772. In our previous analysis of WT Tor autophosphorylation, the presence of the phosphopeptide in peak 1a was obscured by the P-Y site present in peak 1 (P-Y630). However, when Y630 was mutated, peak 1a was observed in Y630F and YY630 + 918FF Tor proteins (19). To further identify this P-Y site, we used the Tor$^{Delta\text{-}Insert}$ protein and automated Edman degradation to determine that the peptide present in peak 1a was phosphorylated on the third and eighth residues after the trypsin cleavage site. These phosphorylated residues previously had been observed as additional ^{32}P peaks released during the sequence analysis of peak 1 from WT Tor (see ref. 19 and Fig. 1*B*). From this data, we conclude that peak 1a is caused by phosphorylation of Y767 and Y772 because they are the only tyrosines in Tor that are three and eight amino acid residues after a trypsin cleavage site. Y767 and Y772 are located in the activation loop of the kinase domain, a region comprising subdomains VII and VIII of the catalytic core, and represent autophosphorylation sites common in RTKs (4). To

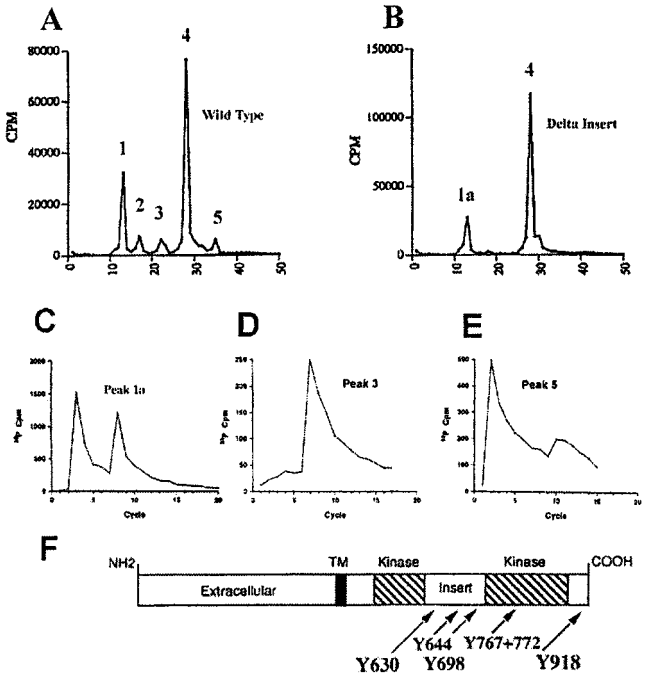

FIG. 1. Identification of Tor autophosphorylation sites. (*A* and *B*) *In vitro* phosphorylated Tor^{WT} and Tor$^{Delta\text{-}Insert}$ were separated by reverse phase HPLC, and fractions were collected. The relative amount of ^{32}P radioactivity in each fraction is shown. Peaks 1 + 1a (*C*), peak 3 (*D*), and peak 5 (*E*) were subjected to sequencing. A summary of the phosphotyrosine analysis is shown in *F*.

confirm our analysis, we generated a mutant Tor protein containing tyrosine to phenylalanine substitutions at positions 767 and 772 (YY767 + 772FF). When peak 1/1a derived from the mutant protein was analyzed by N-terminal sequencing, the ^{32}P signal released at cycle four (corresponding to Y630) was detected, but the signal at cycles three and eight were missing (data not shown). Together, these results identify tyrosines 767 and 772 as autophosphorylation sites of the Tor RTK.

Identification of Phosphotyrosine Sites Y644 and Y698. As discussed above, it is likely that the peptide isolated in peak 3 contains one of the four tyrosines (Y630, 644, 656, and 698) found in the kinase insert region. N-terminal sequence analysis of this sample revealed that the peptide isolated in peak 3 was phosphorylated on the seventh residue after the trypsin cleavage site (Fig. 1*D*). Analysis of the Tor kinase insert sequence, combined with the fact that trypsin does not efficiently cleave Arg-Glu or Lys-Glu bonds, suggests that the peptide isolated in peak 3 most likely comprises residues 692–710 (i.e., IFENKEYFDCLDSS-DTKPR), which contains Y698 in the seventh position. Because there is no other complete or partial tryptic peptide in Tor that contains a tyrosine residue at the seventh position, we conclude that Y698 is the autophosphorylation site contained in peak 3.

Analysis of the Tor$^{Delta\text{-}Insert}$ protein (Fig. 1*B*) suggests that the phosphorylated residue present in peak 5 is also located in the kinase insert region. N-terminal sequencing of the peptide contained within peak 5 indicates that a primary peak of ^{32}P counts is released in cycle 2 whereas a secondary peak is released in cycle 10 (Fig. 1*E*). There are two Tor peptides that contain a tyrosine residue in the second position after a potential trypsin cleavage site (i.e., Y644 and Y698), and both peptides are found in the insert region. We conclude that the major peptide present in peak 5 contains Y644 and comprises residues 643–659 for the following reasons. First, trypsin should efficiently cleave the sequence Lys642-Gly643, but would not be expected to efficiently cleave the sequence Lys696-Glu697. Second, the Y644-containing peptide is predicted to elute much later from the C-18 column than is the Y698-containing peptide (peak 5 versus 3). Third, the release of counts in cycle 10 is consistent with a partial tryptic peptide that comprises residues 635–659, which contains Y644 in the 10th position. Taken together, our results suggest that Y644 is an autophosphorylation site found within the Tor kinase insert region.

The disappearance of peak 2 from the Tor$^{Delta\text{-}Insert}$ protein (Fig. 1*B*) suggests that the peptide present in this peak may also contain a P-Y site found in the kinase insert region. However, we have been unable to obtain conclusive sequencing data from this peak. It is therefore possible that peak 2 may contain P-Y656 or may represent a partial tryptic peptide containing one of the three other tyrosine residues found in the kinase insert region.

Phosphotyrosines in the Tor Insert Domain Are Required for Transmitting a Positive Signal. To determine the functional role of the tyrosine residues located in the Tor kinase insert region (Y630, Y644, Y656, Y698), we examined *in vivo* the signaling activities associated with the corresponding *tor* mutants. Individual tyrosines in the Tor receptor were changed to phenylalanines by site-directed mutagenesis. Point mutants were generated in an 11.5-kilobase *tor*$^+$ genomic fragment that fully rescues *tor* lof mutations (see *Materials and Methods*). The function of the Tor mutant receptors was examined in the background of *tor*^{XR1}, a protein null allele (23, 25), to completely eliminate other sources of Tor receptor. The signaling abilities of the Tor mutant proteins were analyzed by measuring the spatial expression domains of *tll* and *hkb* as well as examining the embryonic cuticles. The WT control P-element fully rescues the terminal phenotypes associated with *tor* ^{XR1} (Table 1). We analyzed embryos derived from *tor*^{XR1} homozygous mothers expressing either one or two copies of the various *tor* transgenes to assess the contribution of the level of expression to the observed phenotype (see Table 1). In these experiments, multiple transformant lines for each transgene were established to control for position effects.

Previous examination of embryos derived from females that express Tor^{Y630F} revealed that Y630 is involved in positive signaling from Tor RTK (19). Here, to test the effect of removing the P-Y644 site, we generated flies carrying a Tor^{Y644F} P-element

Table 1. Signaling activities of Tor mutants *in vivo*

		tll expression		*hkb* expression		Hatching rate	
		n	Percent of egg length	*n*	Percent of egg length	*n*	Percent hatched
Wild type		44	14.8	30	8.2	227	98
tor$^+$	1 copy	45	14.7	48	8.3	198	90
	2 copies	51	14.8	39	8.2	450	93
tor^{Y630F}	1 copy	39	12.5	49	5.8	388	45
	2 copies	49	12.4	53	5.6	318	47
tor^{Y644F}	1 copy	41	14.8	46	8.1	365	95
	2 copies	37	14.9	39	8.2	250	94
tor^{Y656F}	1 copy	47	14.9	41	8.2	455	92
	2 copies	41	14.8	43	8.3	430	90
tor^{Y698F}	1 copy	41	14.7	38	8.3	205	95
	2 copies	45	14.8	49	8.2	360	94
tor^{Quad}	1 copy	29	0	41	0	225	0
	2 copies	33	0	31	0	200	0
tor^{Quint}	1 copy	30	0	27	0	230	0
	2 copies	29	0	41	0	225	0
tor$^{Y767+772F}$	1 copy	38	0	49	0	350	0
	2 copies	41	0	44	0	260	0
tor^{XR1} (lof)		45	0	48	0	195	0

The spatial domain of expression of *tll* and *hkb* was measured in WT and various *tor* mutants. *n* indicates the number of embryos scored in each experiment. The domain of expression of both *tll* and *hkb* was detected as the percent of egg length, and the standard deviation for each sample size is indicated. Hatching rates were determined as the number of hatching larvae divided by the total number of fertilized eggs.

in the *tor*^{XR1} background. Embryos derived from those females exhibited a WT phenotype as measured by their hatching rate (98%) and cuticle structure (Fig. 2*B3*). In addition, the domain of *tll* and *hkb* expression was measured at 14.8 and 8.1% egg length, respectively (Fig. 2 *B1* and *B2*), which is identical to the expression of these genes in WT embryos. Similarly, the function of the P-Y698 site was analyzed by examining the development of embryos derived from Tor^{Y698F} females. Again, all embryos (95%) hatched, and the *tll* and *hkb* expression patterns, in addition to the cuticle phenotype, were WT. The domains of *tll* and *hkb* expression in these embryos were 14.7 and 8.2% egg length (Fig. 2 *C1* and *C2*), respectively (Fig. 2*C3*). To provide a complete analysis of all tyrosine residues located in the Tor kinase insert region, transgenic lines expressing the Tor^{Y656F} receptor also were generated. Embryos derived from females carrying this mutant receptor all hatched and were indistinguishable from WT embryos.

To more fully evaluate the functional role of the tyrosine residues in the kinase insert region, a construct was generated in which all tyrosine residues within the insert region were mutated (Quadruple = Tor$^{YYYY630+644+656+698FFFF}$). All embryos derived from females expressing this construct died during embryogenesis and showed a complete Tor lof phenotype both at the level of *tll* and *hkb* expression and cuticle morphology (Fig. 2 *D1–D3*). To confirm that the mutant protein was expressed, we analyzed Quadruple embryos for Tor protein expression. Lysates obtained from Quadruple embryos were incubated with anti-Tor antibodies, and immunoprecipitates were subjected to Western blot analysis. Blots probed with anti-Tor antibody demonstrated that the mutant Tor protein was expressed in Quadruple embryos. In addition, the mutant protein retained autokinase activity and was phosphorylated on the remaining P-Y sites (Fig. 3 and data not shown). The phenotype associated with the Quadruple mutant is identical to the complete loss of Tor activity and is much stronger than the phenotype observed when the Y630 autophosphorylation site is mutated (19). The Tor lof phenotype of the Quadruple mutant receptor indicates that all of the tyrosine residues present within the kinase insert region, or a subset of these residues, are necessary for a positive signal to be generated from Tor. Because Y630 transduces part of Tor signaling activity (19) and Y656 is not phosphorylated, we propose that a combination of Y630 + Y644, Y630 + Y698, or Y630 + Y644 + Y698 is needed for the receptor to achieve full signaling activity. Overall, our analysis illustrates both the redundancy and synergistic interactions between phosphotyrosine sites in Tor signaling.

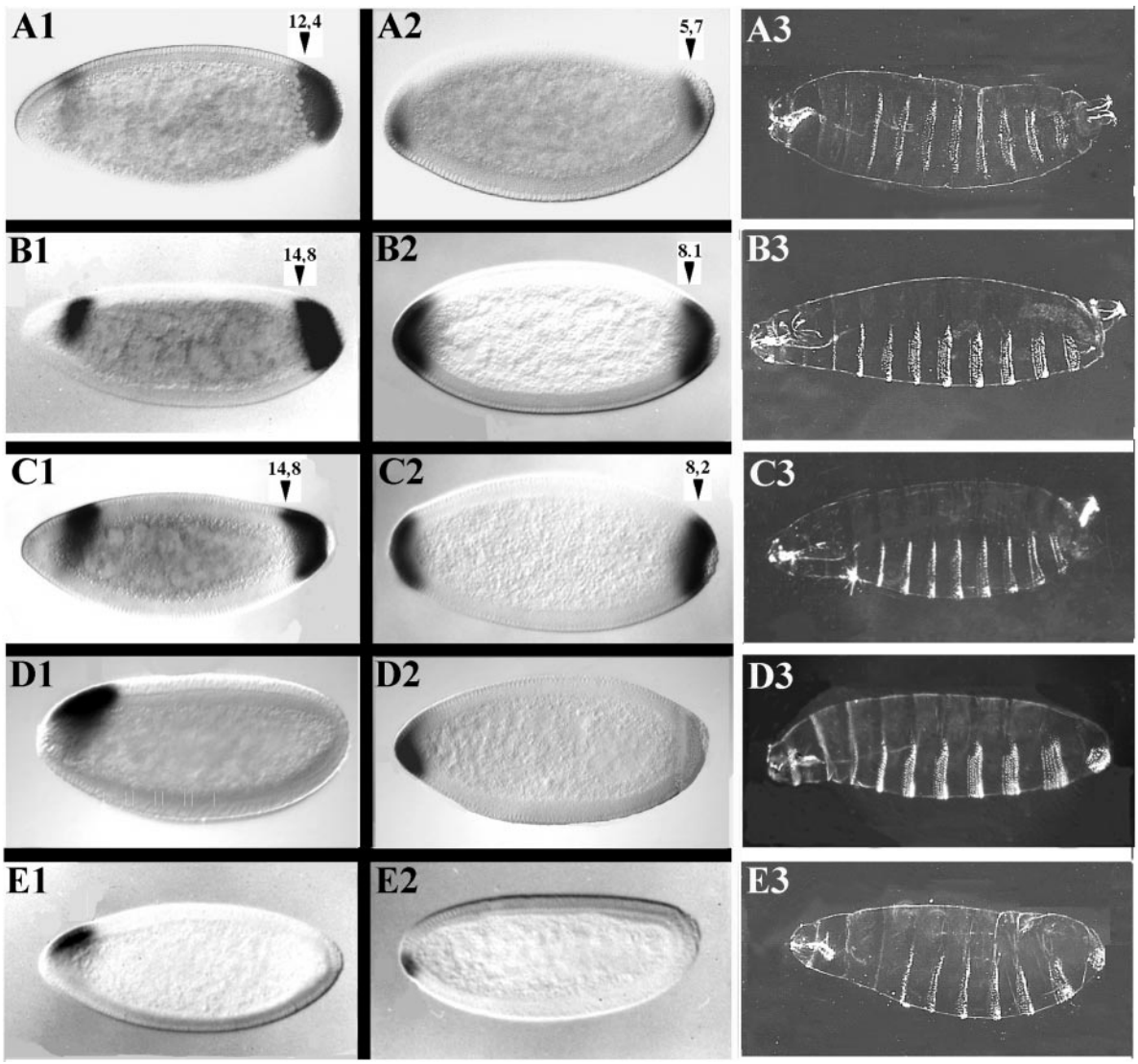

FIG. 2. Effect of Tor tyrosine mutations on embryonic development. The effect of the *tor* P-Y mutations on the expression of *tll* (*A1*, *B1*, *C1*, *D1*, and *E1*) and *hkb* (*A2*, *B2*, *C2*, *D2*, and *E2*) and cuticle phenotypes (*A3*, *B3*, *C3*, *D3*, and *E3*) are shown. The mutations shown are: Tor^{Y630F} (*A*), Tor^{Y644F} (*B*), Tor^{Y698F} (*C*), Tor^{Quadruple} (*D*), and Tor$^{Y767+772F}$ (*E*). The effect of these mutations was analyzed in the *tor*^{XR1} (Tor protein null) mutant background. The domain of *tll* and *hkb* expression are indicated as percent egg length, with 0% corresponding to the posterior pole. Tor^{Y630F} shows a partial lof phenotype; Tor^{Y644F}, Tor^{Y656F}, and Tor^{Y698F} show a WT phenotype; and the Tor^{Quadruple} and Tor$^{Y767+772F}$ show a lof phenotype.

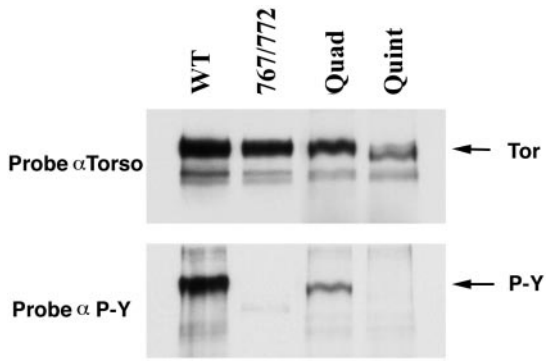

FIG. 3. Expression and tyrosine phosphorylation of mutant Tor proteins. Lysates were prepared from embryos expressing various Tor mutant proteins. Tor proteins were immunoprecipitated, and the precipitates were examined by immunoblot analysis using antibodies recognizing Tor (αTor). Blots then were stripped and reprobed with antibodies recognizing phosphotyrosine (αP-Y). The position of Tor and P-Y Tor is indicated on the right by an arrow.

Role of Y918 in Regulation of Tor Activity. In addition to the P-Y sites found in the kinase insert region, the P-Y918 site lies in a region (C-terminal tail) outside of the core catalytic domain of Tor (19). Our previous studies revealed that elimination of P-Y918 in an otherwise WT receptor leads to hyperactivation of Tor (ref. 19; Fig. 4). More recently we have found that the *Drosophila* homolog of RasGAP associates with P-Y918 and is a negative effector of Tor signaling. In addition Csw, the positive effector of P-Y630, specifically dephosphorylates P-Y918 (21). To determine the effect of mutating all P-Y sites outside the core catalytic domain, we analyzed the activity of a Quintuple (YYYYY630 + 644 + 656 + 698 + 918FFFFF) Tor mutant *in vivo*. We found that this receptor was unable to transmit the Tor signal (Table 1). Thus, even when the Y918 site is mutated, phosphorylation of some tyrosine sites within the kinase insert region is still needed for Tor signaling. Of interest, although the Quintuple mutant protein was expressed in embryos, it did not show any detectable tyrosine phosphorylation (Fig. 3).

***In Vivo* Function of Y767 and Y772.** Next, we examined the functional role of the P-Y sites (Y767 and Y772) found in the activation loop of the Tor kinase domain. Transgenic flies containing the YY767 + 772FF mutant were generated, and the activity of this mutant receptor was examined in the Tor null

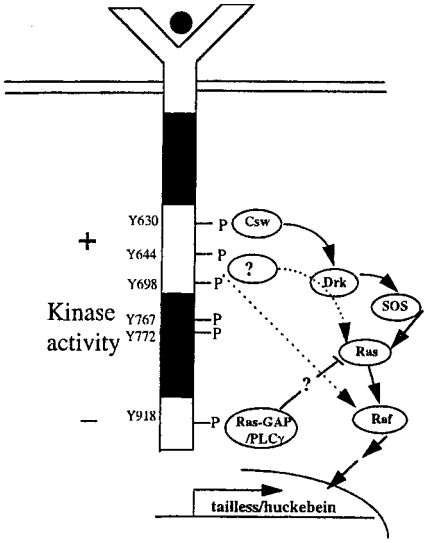

FIG. 4. Tor tyrosine phosphorylation and its function in mediating the developmental signal to downstream molecules. See text for details.

background as described above. All embryos derived from females expressing YY767 + 772FF Tor died during embryogenesis and exhibited a clear *tor* lof phenotype in terms of *tll* and *hkb* expression and cuticle morphology (Fig. 2 *E1–E3*). To confirm that the mutant Tor protein was expressed, lysates obtained from YY767 + 772FF embryos were incubated with anti-Tor antibodies, and immunoprecipitates were subjected to Western blot analysis. Blots probed with anti-Tor antibody showed that YY767 + 772FF embryos express a Tor protein similar in size to WT Tor; however, probing the blot with anti-P-Y antibody revealed that the mutant protein contained no detectable tyrosine phosphorylation (Fig. 3). These results suggest that the P-Y767 and 772 sites located in the kinase domain activation loop are required for the *in vivo* autokinase activity of Tor and that the elimination of these sites leads to a Tor lof phenotype.

DISCUSSION

Roles of Phosphotyrosine Residues in Tor Signaling. Our analysis reveals that Tor autophosphorylates on tyrosine residues located in both noncatalytic (the kinase insert region and the C-terminal tail) and catalytic regions (the activation loop) of the molecule. In the kinase insert, we were able to demonstrate that three (Y630, Y644, and Y698) of the four tyrosines present are phosphorylated. Mutation of individual P-Y sites either had no effect on or reduced the level of Tor signaling whereas the simultaneous mutation of all four tyrosines (Y630, Y644, Y656, and Y698) eliminated Tor signaling. These results demonstrate that the tyrosine residues within the kinase insert domain are essential for Tor signal transduction and that multiple P-Y sites act synergistically to propagate the Tor signal. The P-Y630 site serves as a binding site for Csw; however, it is not yet known what molecules bind to either P-Y644 or P-Y698. Of interest, there appears to be redundancy between the Y644 and Y698 sites because neither mutation of Y644 or Y698 alone is associated with a decrease in Tor activity. Finally, Y656, which is located within the insert region of Tor, does not appear to be phosphorylated and has no apparent activity in triggering downstream signaling events. Thus, we propose that Tor positive signaling is transduced by the synergistic activities of Y630 with Y644 and/or Y698.

To date, the only members of the Tor pathway that contain SH2 domains are Csw and Drk. Csw, the *Drosophila* homolog of SHP-2, encodes a nonreceptor tyrosine phosphatase with two N-terminal SH2 motifs (20). Drk, the *Drosophila* homolog of Grb2, encodes an adapter protein containing one SH2 and two SH3 motifs and functions to recruit the exchange factor Sos to the activated RTK (33, 34). Previously, we reported that Csw associates directly with P-Y630 and that there is no direct binding sites for Drk on Tor (19). More recently, we have found that Csw has at least two distinct functions in Tor signaling (21). First, it regulates positive signaling through Drk because P-Y666 on Csw is a Drk-binding site. Second, it blocks the activity of a negative regulator of Tor signaling that binds to the P-Y918 site. Mutation of Y918 leads to an increase in Tor activity (19), and we have found that P-Y918 is a binding site for a *Drosophila* Ras-GAP protein that contains two SH2 motifs (21). Csw dephosphorylates P-Y918 and thus prevents the negative regulator RasGAP from associating with Tor.

We envision three models by which P-Y644 and P-Y698 residues transduce the Tor signal. In the first model, these P-Ys may bind an adapter molecule(s), which would recruit either Csw and/or Drk to Tor. This model predicts that activation of all downstream signaling events is mediated solely by Csw and Drk, a hypothesis that can be tested by examining the phenotype of embryos derived from germlines missing both Csw and Drk activities. Possible candidates for such an adapter include SHC and NCK/DOCK (35–37), although it is not known whether these proteins bind Csw/SHP-2 or Drk/Grb2. In a second model, Tor could transduce a signal through activation of Csw and Drk, as well as through Dos. Dos encodes a protein with an amino-

terminal pleckstrin homology domain, a polyproline motif that may bind an SH3 domain, and 10 potential P-Y sites with consensus sequences for binding SH2 domains (15). Previous studies have implicated Dos as a component of the Tor signaling pathway because embryos derived from females that lack maternal Dos activity show a partial lof Tor phenotype (15), and studies by Herbst *et al.* (38) demonstrated the ability of Dos to bind Grb2 and Csw. In the third model, P-Y644 and P-Y698 could mediate activation of the Raf kinase in a pathway that does not require Ras1 activity. Previous work has suggested the existence of a Ras1-independent pathway of Raf activation (13). In this scenario, a novel, yet unidentified protein could bind to Y644 and Y698, through either SH2 domain or PTB domains, and could lead to Raf activation in a Ras1-independent manner.

Finally, we have documented a role for the phosphorylation of Y767 and Y772, two tyrosine residues located in the activation loop of the kinase domain. Mutation of these P-Y sites completely eliminated Tor signaling. This phenotype is likely caused by the effect that these mutations have on Tor catalytic activity itself. First, catalytic activity has been shown to be essential for Tor signaling (23). Second, we were unable to detect any tyrosine phosphorylation of the Tor protein isolated from embryos expressing $Tor^{YY767+Y772FF}$, and tyrosine phosphorylation of $Tor^{YY767+Y772FF}$ expressed in Sf9 cells was greatly reduced (at least 50-fold) when compared with the WT protein. Third, autophosphorylation of the corresponding tyrosine residues in the insulin receptor (39), the scatter factor/hepatocyte growth factor receptor (40, 41), the nerve growth factor receptor (42, 43), and the fibroblast growth factor receptor (44) has been shown to be required for catalytic activity of these kinases. Based on crystal structure analysis of the fibroblast growth factor receptor (45) and the insulin receptor (46), autophosphorylation of tyrosine residues in the activation loop results in a dramatic change in conformation, thus relieving an autoinhibitory mechanism and allowing unrestricted access to the binding sites for ATP and protein substrates. Here, we provide data in trangenic animals supporting the role for the autophosphorylation of activation loop residues in RTK signaling.

Comparison with Other RTKs. It is of interest to compare our findings on the role of noncatalytic P-Y residues in Tor signaling with structure/function analyses of other RTKs, such as the PDGF, EGF, and Sev RTKs. Our results indicate that P-Y autophosphorylation sites are essential for Tor-mediated signal transduction, consistent with the role that P-Y residues play in PDGF receptor signaling but in contrast to that observed for both the Sev and EGF RTKs. Because a PDGFβ receptor lacking five P-Y autophosphorylation sites fails to relay a mitogenic signal, the interaction between the PDGF receptor and SH2 domain-containing proteins has been determined to be essential for signal transduction. By adding back individual P-Y sites, PDGF receptor mutants capable of binding only one SH2 domain-containing molecule have been generated, thus making it possible to distinguish between independent parallel pathways leading to DNA synthesis (47). The Sev RTK, on the other hand, contains only one P-Y site (P-Y2546) located outside of the kinase domain, and this site has been shown to interact with Drk. A Sev receptor mutated at P-Y2546 retains activity (48), suggesting that the residual signaling activity is mediated by an indirect interaction between Drk and Sev or by a Drk-independent parallel pathway. Similarly, mutagenesis of the mammalian EGF receptor (14, 49) as well as the *C. elegans* homolog Let-23 (50) have revealed that, when all possible SH2- or PTB-binding sites are eliminated, these receptors still retain activity. In the case of the mammalian EGF receptor, a receptor missing all known autophosphorylation sites was unable to bind SH2-domain containing proteins; however, it was fully competent to stimulate gene expression, MAP kinase activation, and mitogenesis. This mutant EGF receptor is believed to compensate for direct SH2 or PTB domain binding by phosphorylating other cellular proteins, primarily SHC, which then serves as an adapter for SH2 containing proteins (49).

In conclusion, although the importance of autophosphorylation of RTKs after ligand binding and activation of the kinase activity is apparent, the degree to which it is needed for mediating a developmental signal varies. In the case of the Tor receptor, autophosphorylation is essential because the elimination of all positive phosphotyrosine sites leads to a Tor lof phenotype. However, in the case of other RTKs, additional mechanisms have been deployed during evolution to regulate downstream signaling.

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