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Phosphorylation site specificity of the Pak-mediated regulation of Raf-1 and cooperativity with Src

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Abstract The p21-activated kinase, Pak, has recently been shown to phosphorylate Raf-1 on serine 338 (S338), a critical regulatory residue. The specificity requirements for Pak-mediated phosphorylation of S338 were examined by substitution analysis of Raf-1 peptides and conserved region 3 (CR3) proteins. Phosphorylation was found to be very sensitive to alterations in amino acid side chains proximal to S338. Loss of N-terminal arginines resulted in decreased peptide phosphorylation while loss of these residues, as well as C-terminal glutamates and bulky C-terminal hydrophobic residues, decreased phosphorylation of the CR3 protein. Phosphorylation of Raf-1 on tyrosine 341 is significant in epidermal growth factor- and Src-mediated signaling, suggesting that cooperativity may exist between Pak and Src phosphorylation of Raf-1. Purified Pak and Src were found not to be cooperative in phosphorylating peptides or purified CR3 protein. However, the phosphorylation of Raf-1 S338 by Pak was increased in the presence of Src. The complexity of this signaling module could thus account for the different levels of Raf-1 activation required for fulfillment of different biological roles within the cell. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Raf-1; Ras; p21-activated protein kinase; Src; Protein kinase; Specificity; Cooperativity; Phosphorylation

1. Introduction

Regulation of the Raf-1 protein kinase is complex and involves many protein interactions and post-translational modifications [1]. Following exchange of GTP for GDP the membrane-associated GTPase, Ras, recruits Raf-1 to the plasma membrane [2]. Raf-1 then undergoes a number of changes in phosphorylation state and protein interactions [3–6]. Major sites of phosphorylation on Raf-1 are serines 43, 259 and 621 [7,8]. A number of recent studies have demonstrated that phosphorylation of serine 338 (S338) and tyrosine 341 (Y341) are critical for mitogenic signaling in response to certain stimuli [2,9–12]. The proximity of these two sites has suggested that cooperativity may exist between the two protein kinases responsible for phosphorylating this region [6,12]. While tyrosine phosphorylation has been studied in the context of Src, we recently demonstrated the p21-activated kinase, Pak3, as capable of phosphorylating S338 [13]. We have subsequently extended this observation to Pak1 and Pak2 (unpublished data). This observation linked another signaling pathway into the complex regulatory mechanism of Raf-1 activation, namely that acting through phosphatidylinositol 3-kinase, Dbl-family guanine nucleotide exchange factors and Cdc42/Rac [14,15].

While the regulation and activity of the p21-activated kinases in biological systems are complex matters in their own right [16], some studies have examined the substrate specificity of these kinases. Pak2 has been shown to phosphorylate intact endothelial cell non-muscle myosin II and isolated recombinant myosin regulatory light chains, but not myosin heavy chains [17]. This phosphorylation is dependent upon the presence of arginine at the P−3 position. Interestingly, the myosin I heavy chain kinase has been shown to be a distinct Pak-family kinase [18] and phosphorylates myosin I heavy chains in a manner similar to that observed for Pak1 [19]. Substrate specificity of Pak2 has been examined by substitution analysis of peptides based on the Rous sarcoma virus nucleocapsid protein sequence KKRKSGL [20]. This group found a requirement for basic amino acids in the P−2 and P−3 positions, quoting the optimal minimal sequence as (K/R)RXS, where X is any acidic, basic or neutral amino acid. Acidic or basic amino acids at P−1 and P−4 increase the efficiency of phosphorylation, while proline at P−1 or P+1 diminishes this activity. Basic residues have also been implicated in Pak1 specificity [21]. Arginines at P−3 to P−5 and especially at P+2 in the ribosomal protein S6 peptide 229–244 (AKRRRLSSLRASTSKS) are essential for efficient phosphorylation of serine 236. Substitution of serine 235 dramatically raises the K_m, indicating sensitivity to proximal residues.

In this study we examined the substrate site specificity of Pak centered on Raf-1 S338. In addition, the extent of cooperativity was measured between Pak and Src in the phosphorylation of the Raf-1 S338Y341 site. Direct and indirect mechanisms of cooperation were examined in cells as well as in vitro using purified proteins.

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Abbreviations: Pak, p21-activated protein kinase; CR3, conserved region 3 of Raf-1; TGF, transforming growth factor

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2. Materials and methods

2.1. DNA constructs and proteins

Constitutively active (F91S, G93A, P95A) and dominant-negative (K297R) hemagglutinin (HA)-tagged murine Pak3 constructs in pJ3H were gifts of S. Bagrodia and R.A. Cerione. Constitutively active Src (Y527F) in pECE-A was supplied by G.S. Feng and dominant-negative Src (K296R, Y528F) in pUSEamp was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Human cRaf-1, cloned into pEXV-3 with an N-terminal FLAG-epitope tag, was generously provided by T.W. Sturgill. The Promega (Madison, WI, USA) Altered Sites pALTER vector was used for mutagenesis of human Raf-1 conserved region 3 (CR3; residues 307–648) as per the manufacturer's protocol. Mutants were then excised with *Eco*RI and *Xho*I and subcloned into the Stratagene (La Jolla, CA, USA) pCal-n vector for expression in BL21 (pLysS DE3) as N-terminal calmodulin-binding peptide fusion proteins (CBP–CR3). Proteins were induced at 30°C with 0.5 mM isopropyl-β-D-thiogalactoside and recovered using the manufacturer's protocol. Histidine-tagged mouse Pak3 was prepared from *Sf*9 cells and was kindly provided by S. Bagrodia and R.A. Cerione.

2.2. Cell culture, transfection and lysis

COS-7 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) with high glucose (Biowhittaker, Walkersville, MD, USA) and supplemented with 5% fetal calf serum, 5% calf serum, sodium pyruvate, L-glutamine and penicillin–streptomycin. Cells were grown in 10% carbon dioxide at 37°C. Cells were transfected by electroporation with 10 μg of each construct, as described elsewhere [22]. Blank pEXV-3 vector and sheared salmon sperm DNA were used as controls. Cells were harvested on the third day after transfection by lysis in 50 mM Tris–HCl, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml each of aprotinin, leupeptin and pepstatin A, pH 8. Lysates were aspirated through a 25-gauge needle four times and cell debris was pelleted by centrifugation at $100\,000 \times g$, 4°C for 15 min. HA-tagged Pak was immunoprecipitated from cells lysed in buffer (above) for 20 min on ice. Immunoprecipitations were performed at 4°C with 2-h primary antibody incubations followed by 1 h with Gamma-Bind Plus Sepharose beads (Pharmacia, Piscataway, NJ, USA). Immunoprecipitates were washed three times with lysis buffer and once with kinase assay buffer prior to assay. Constitutively active Src (Y527F) was recovered from Src-transformed Rat2 fibroblasts, generously provided by G.S. Feng. Src was immunoprecipitated with an anti-Src antibody (Santa Cruz, CA, USA), washed three times with RIPA buffer and once with kinase buffer prior to assay.

2.3. Kinase assays

Raf-1 kinase activity in anti-FLAG immunoprecipitates was measured by a coupled-kinase method described by Stokoe et al. [23]. Activities were normalized to the level of FLAG-Raf-1 present in each immunoprecipitate determined by anti-FLAG Western blotting of the primary incubations. In vitro kinase assays used a Raf-1 peptide or CBP–CR3 substrates. Peptides corresponding to wild type or substituted Raf-1 residues 331–345 (Table 1) were incubated at final concentrations of 500 μM with recombinant Pak3, or active Src, in the presence of 500 μM ATP, containing 4–6 μCi [γ-^{32}P]ATP, at 30°C for 15 min. Incubations were then spotted onto P-81 phosphocellulose papers (Whatman, Fairfield, NJ, USA). The P-81 papers were washed repeatedly in 75 mM phosphoric acid, once in acetone, air-dried and counted in 5 ml of liquid scintillant. Wild type and mutant CBP–CR3 proteins ($\sim$1 μg each, see Table 1) were incubated with recombinant Pak3 in the presence of 500 μM ATP, containing 6–10 μCi [γ-^{32}P]ATP, at 30°C for 20 min. Incubations were resolved by SDS–PAGE and transferred to Immobilon-P (Millipore, Bedford, MA, USA). Membranes were then stained with 0.25% Ponceau S in 5% acetic acid, dried and autoradiographed with Hyperfilm-MP (Amersham, Piscataway, NJ, USA). Radiolabeled bands were aligned with stained protein bands to verify phosphorylation of CBP–CR3 and levels of phosphorylation were measured by densitometric scanning of autoradiographs as above.

Pak3 kinase activity was determined from duplicate HA-Pak3 immunoprecipitations from transfected cells. Immunoprecipitates were washed, on ice, three times with lysis buffer and once with assay buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EGTA, 0.02% Brij 35 and 0.5% β-mercaptoethanol). The immunoprecipitates were then incubated in assay buffer containing 500 μM ATP, 10 μCi [γ-^{32}P]ATP and 500 μM Raf-1 peptide substrate, for 30 min at 30°C. Reactions were terminated by transferring onto P-81 filter paper as described above and radioactivity associated with the dried filters was determined by scintillation counting.

2.4. Western blotting

Western blotting was used to quantitate the presence of Raf-1 in immunoprecipitates, using an anti-FLAG antibody (mAb M2, Kodak/Sigma, St. Louis, MO, USA). Pak protein expression was verified using an anti-HA antibody (Santa Cruz). Neither Src construct was tagged, but expression was confirmed by blotting with anti-Src (Santa Cruz) to show increased levels of expressed Src protein in lysates. Phosphorylation of Raf-1 on S338, or S338 and Y341 together, was visualized by immunoprecipitation with phospho-specific antisera (Research Genetics, Huntsvillle, AL, USA) followed by immunoblotting with anti-FLAG antibody. Values from densitometric scanning of these blots corresponding to FLAG-Raf-1 were then normalized for total FLAG-Raf-1 expression with densitometer readings from parallel anti-FLAG blots of cell lysates.

3. Results

3.1. Phosphorylation of Raf-1 peptides

Synthetic peptides corresponding to Raf-1 residues 331–345 were used as substrates for Pak3. In addition to the wild type, a range of substituted peptides was generated (Table 1). Selected residues were altered to remove phosphorylation sites, charges, reduce side chain steric constraints or mimic prior phosphorylation by substitution with acidic residues (Fig. 1A). Dual substitution of S338/S339 with either alanine or acidic residues obliterated the ability of Pak3 to phosphorylate the substrate in agreement with our previous cell culture data [13]. Dual substitution of Y340/Y341 with phenylalanine resulted in only a marginal reduction in peptide phosphorylation. Substitution of aromatic residues 340–342 with alanines increased peptide phosphorylation by Pak3 by about 30%, which may result from S338 being more sterically accessible to the kinase. Substitution of Y340/Y341 with aspartates,

Table 1
Substitutions in Raf-1 examined in this study

	Substitution	Type
Wild type (WT)	RPRGQRDSSYYWEIE	n/a
S338T	-------T-------	S-T
S338A	-------A-------	PKO
S339A	--------A------	PKO
S338A, S339A	-------AA------	PKO
Y340F	---------F-----	PKO
Y341F	----------F----	PKO
Y340F, Y341F	---------FF----	PKO
S338D	-------D-------	PM
S339D	--------D------	PM
S338D, S339E	-------DE------	PM
Y340D	---------D-----	PM
Y341D	----------D----	PM
Y340D, Y341D	---------DD----	PM
D337N	------N--------	CKO
R336Q	-----Q---------	CKO
R333Q, R336Q	--Q--Q---------	CKO
R333Q, R336Q, D337N	--Q--QN--------	CKO
E343Q, E345Q	----------Q-Q	CKO
W342A	----------A---	SKO
Y340A, Y341A	---------AA----	SKO
Y340A, Y341A, W342A	---------AAA---	SKO

Each substitution is listed with a designated function. Key: PKO, phosphorylation site knock-out; PM, phosphorylation mimetic; CKO, charged residue knock-out; SKO, steric residue knock-out.

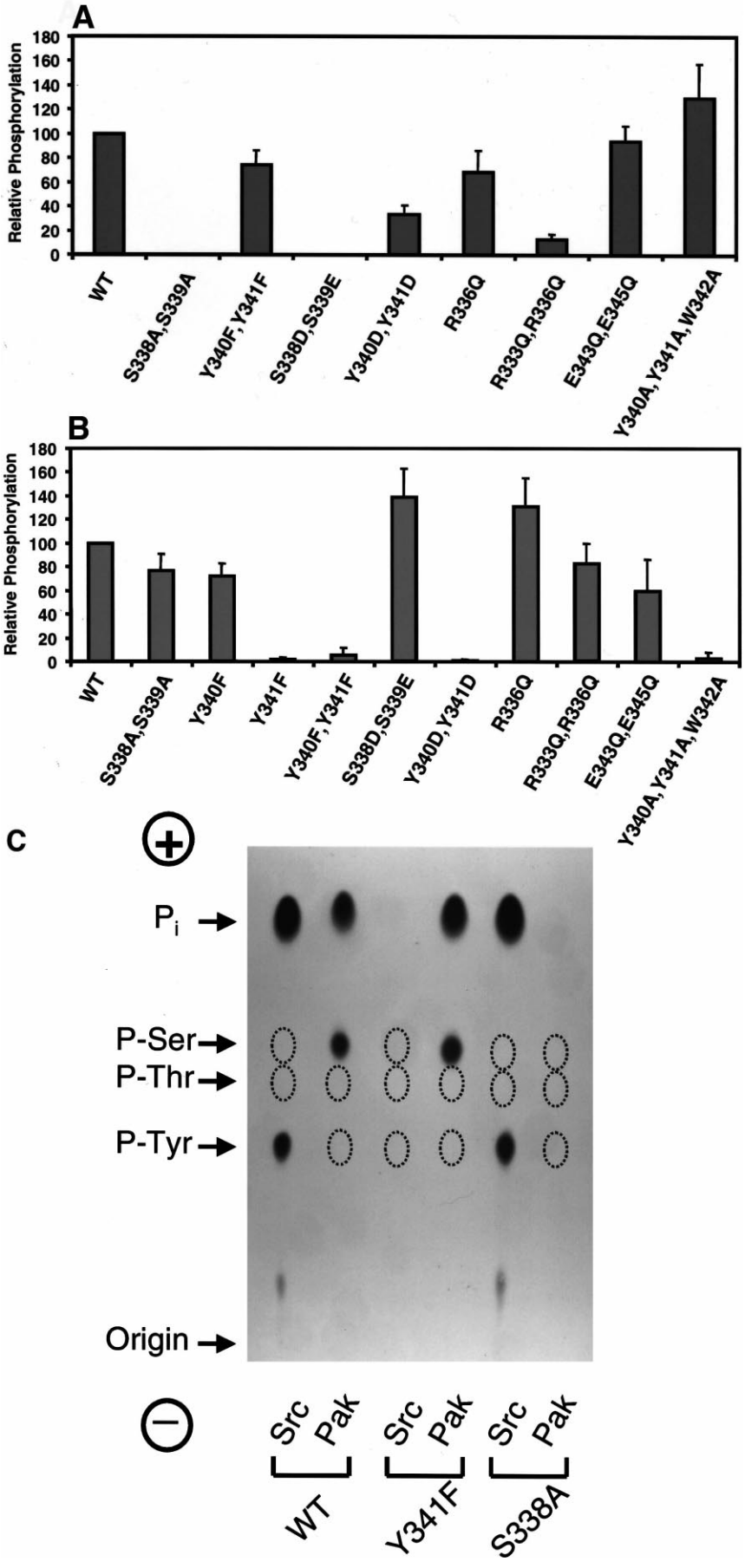
A
Relative Phosphorylation
180
160
140
120
100
80
60
40
20
0
WT
S338A,S339A
Y340F,Y341F
S338D,S339E
Y340D,Y341D
R336Q
R333Q,R336Q
E343Q,E345Q
Y340A,Y341A,W342A
B
Relative Phosphorylation
180
160
140
120
100
80
60
40
20
0
WT
S338A,S339A
Y340F
Y341F
Y340F,Y341F
S338D,S339E
Y340D,Y341D
R336Q
R333Q,R336Q
E343Q,E345Q
Y340A,Y341A,W342A
C
+
Pi
P-Ser
P-Thr
P-Tyr
Origin
−
Src
Pak
Src
Pak
Src
Pak
WT
Y341F
S338A

Fig. 1. Phosphorylation of Raf-1 synthetic peptides by Pak3 and Src. Peptides corresponding to wild type or substituted Raf-1 residues 331–345 (see Table 1) were incubated at final concentrations of 500 μM with (A) recombinant Pak3 or (B) active Src, immunoprecipitated from S7a cells, in the presence of 500 μM ATP, containing 4–6 μCi [γ-^{32}P]ATP, at 30°C for 15 min. Results are the means of at least four separate experiments. Phosphoamino acid analysis showed site and residue type specificity of Pak3 and Src kinases with respect to Raf-1 peptides (C). Recombinant Pak3 or immunoprecipitated active Src were used to phosphorylate Raf-1 peptides; wild type (WT), S338A, Y341F. Broken ovals indicate the positions of non-radiolabeled phosphoamino acid standards.
←

mimicking prior phosphorylation, had a negative effect (>60% decrease) on S338 phosphorylation. This might imply that the presence of negative charges in close C-terminal proximity to S338 is unfavorable to phosphorylation by Pak3. Substitution of E343 and E345 with glutamine was inconsequential to Pak phosphorylation. However, single glutamine substitution of R336 decreased Pak3-mediated phosphorylation (~30%), while dual glutamine substitution of R333 and R336 decreased peptide phosphorylation >80%. Similar results were obtained with Pak1 and Pak2 (unpublished data).

In contrast to Pak3, immunoprecipitated active Src demonstrated different substrate requirements within the Raf-1 peptide. Src was shown to be specific for Y341 (Fig. 1B), as has been reported elsewhere [12]. Substitution of Y341 with phenylalanine abrogated Src-mediated peptide phosphorylation, while tyrosine substitution of Y340 had little effect. Additionally, Src displayed a different preference for charged residues flanking the Y341 phosphorylation site. Loss of the positive charges at R333 and R336 had little effect on Src phosphorylation of the peptide. However, substitution of E343 and E345 with glutamine decreased Src phosphorylation of Y341 approximately 40%. This agrees with the general specificity of Src family kinases for phosphorylation sites flanked by acidic residues. Substitution of S338S339 with acidic residues caused a modest, yet significant, 40% increase in phosphorylation by Src. This result raises the possibility that prior phosphorylation of S338 might enhance the ability of Src to phosphorylate Y341. Finally, the specificity of Raf-1 peptide phosphorylation by purified Pak3 and Src was confirmed by phosphoamino acid analysis [24] of phosphorylated wild type, S338A and Y341F peptides (Fig. 1C).

3.2. Phosphorylation of Raf-1 CR3 proteins

The Raf-1 CR3 domain represents a more structurally constrained substrate with which to study Pak substrate recognition requirements. Having observed the importance of charges in the peptide, CR3 mutants were generated to test these observations further (Table 1). The relative phosphorylation of CR3 proteins by Pak3 is shown in Fig. 2. What became apparent was that phosphorylation of S338 is exquisitely sensitive to side chain alterations in residues close to S338, including substitution of S338 with threonine. In contrast to the peptide results, loss of aromatic side chains at 340, 341 and 342 resulted in greatly decreased CR3 phosphorylation by Pak3. This effect was greater for residues closer to S338, wherein W342A exhibited the least effect. Interestingly, substitution of Y340 with aspartic acid all but eliminated S338 phosphorylation while substitution of Y341 with aspartic acid was well tolerated. This result predicts that Pak should be able to phosphorylate S338 in the presence of phosphorylated Y341. The presence of a proximal negative charge at position 339 (S339D) was detrimental to peptide phosphorylation. Charged residues were again significant in determination of Pak3-mediated phosphorylation efficiency. Substitution of either R333/R336 or E343/E345 with glutamines had a negative effect on S338 phosphorylation. As with the peptides, R333 and R336 were found to be essential, with D337 being relatively unimportant, in directing Pak3 phosphorylation of S338. In contrast to the peptide results, the presence of negatively charged residues at 343 and 345 was required for phosphorylation.

3.3. Pak and Src show synergy in Raf-1 activation

Cooperativity between Pak3 and Src was studied in transiently transfected COS-7 cells where Raf-1 activation and phosphorylation in vivo were monitored. Raf-1 activity was measured from COS-7 cell lysates following co-transfection with combinations of Pak3 and Src. Combinations of consti-

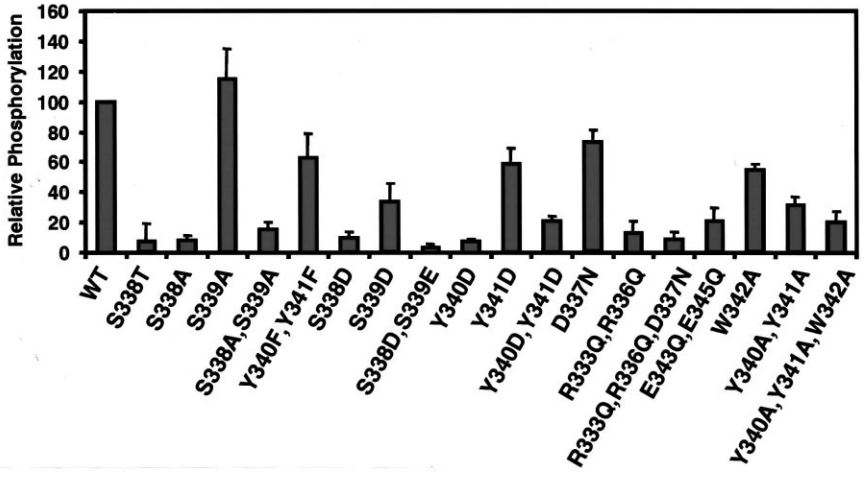

Fig. 2. Phosphorylation of CBP–CR3 fusion proteins by Pak3. Wild type or substituted CBP–CR3 proteins (~1 μg each) were incubated with recombinant Pak3 in the presence of 500 μM ATP, containing 6–10 μCi [γ-^{32}P]ATP, at 30°C for 20 min. Results shown are the means of at least four separate experiments.

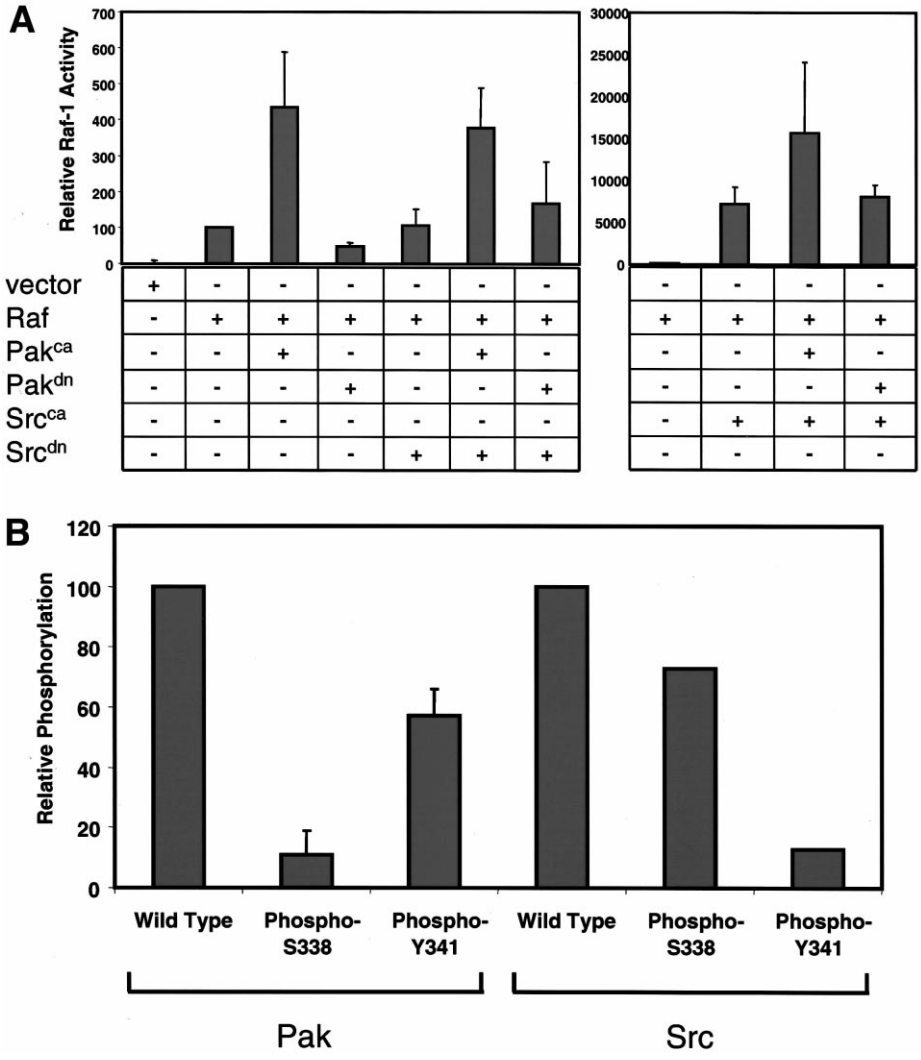

Fig. 3. Study of the potential for Pak3 and Src to cooperate in Raf-1 activation and phosphorylation. A: Analysis of the activation of Raf-1 in COS-7 cells by different combinations of Pak3 and Src constructs. COS-7 cells were transiently transfected with FLAG-tagged Raf-1 and different Pak3 and Src constructs. Cells were harvested 3 days later, Raf-1 was immunoprecipitated and immune complexes were assayed for kinase activity in a coupled assay. B: Examination of the effect of prior phosphorylation of other sites on the ability of Pak3 or Src to phosphorylate S338 or Y341, respectively. Peptides were chemically phosphorylated on selected residues and used for in vitro kinase assays with recombinant Pak3 or immunoprecipitated active Src, as described in Fig. 1. Results shown are the means of four separate experiments.

tutively active Pak3 (Pak^{ca}) or Src (Src^{ca}), or dominant-negative Pak3 (Pak^{dn}) or Src (Src^{dn}) were examined for their effects on Raf-1 activation. While either Pak^{ca} or Src^{ca} was individually capable of eliciting Raf-1 activation, a cooperative effect of Pak^{ca} and Src^{ca} was observed when the kinases were presented together. Moreover, Raf-1 activation by Src^{ca} alone was greater than that achieved by Pak^{ca} alone. Expression of Src^{ca} and Pak^{ca} together resulted in a further increase in Raf-1 activation, illustrating potential cooperativity between the two kinases. Expression of dominant-negative Src did not significantly diminish Raf-1 activation by Pak^{ca} nor did dominant-negative Pak reduce Src^{ca} activation of Raf-1. This result suggests that phosphorylation of either S338 or Y341 alone is sufficient to modulate Raf-1 activity, although the degree of activation elicited by each respective phosphorylation differs by more than an order of magnitude.

3.4. Effect of prior phosphorylation on Pak3 and Src activities

The COS-7 cell experiments suggested that cooperativity may occur between Pak and Src in Raf-1 activation. This could be explained by S338 phosphorylation increasing Src-mediated phosphorylation of Y341 (as suggested by the peptide experiments), or by the sum of the mechanistic contributions of each phosphorylation. We returned to in vitro experiments using Raf-1 peptides corresponding to residues 331–345 to address the question of whether the presence of a phosphate at Y341 or S338 could influence phosphorylation of the peptide by Pak3 or Src, respectively. Raf-1 331–345 peptides were synthesized containing either phospho-S338 or phospho-Y341. The presence of a phosphate at S338 or Y341 resulted in the loss of significant substrate phosphorylation by Pak3 or Src, respectively (Fig. 3B). However, the consequence of having an adjacent phospho-serine did not enhance Y431 phosphorylation by Src nor did the presence of phospho-Y341 enhance S338 phosphorylation by Pak3. This result suggests that while the presence of adjacent phosphoamino acids is compatible with additional phosphorylation of the 338/341 region by Src or Pak, the additional negative charge does

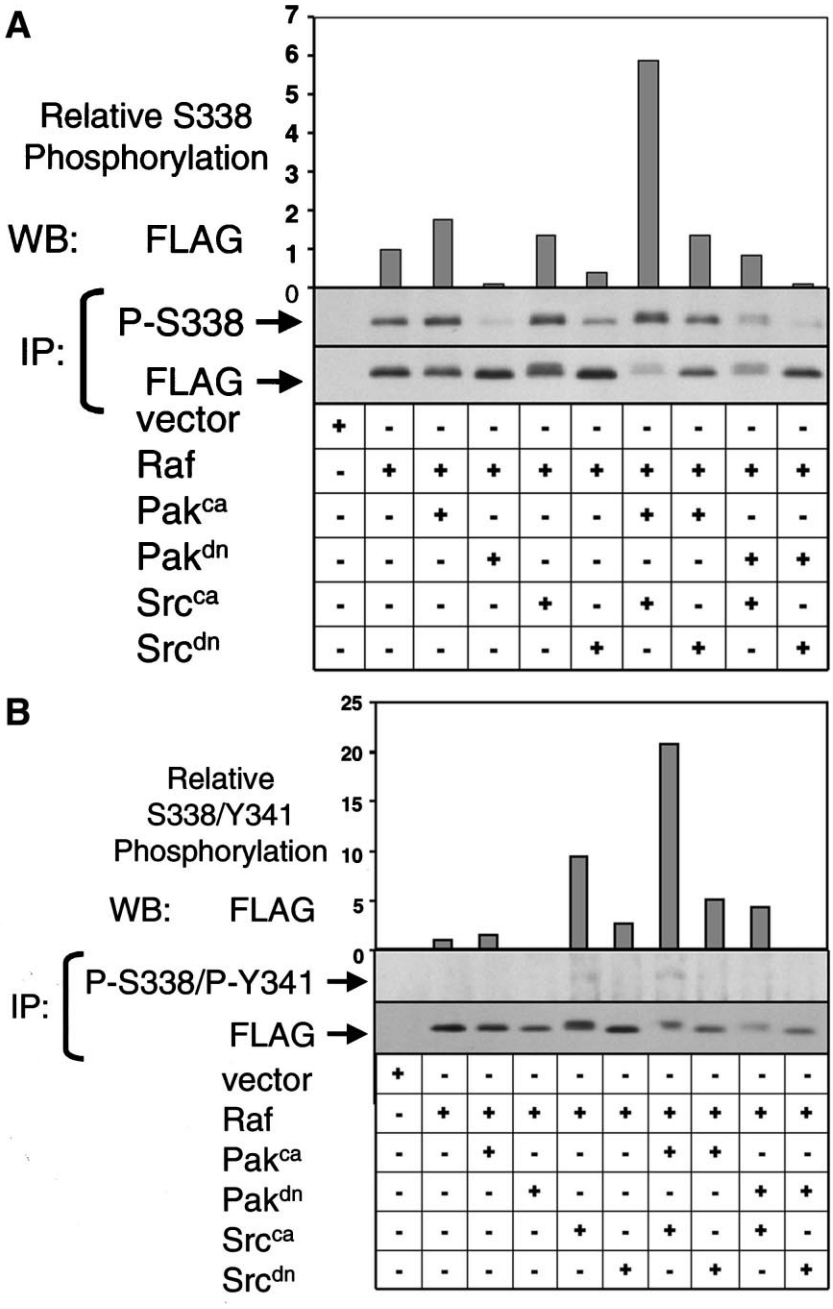

Fig. 4. Analysis in COS-7 cells of the phosphorylation of S338 and Y341 in Raf-1 by Pak3 and Src. COS-7 cells were transiently transfected with FLAG-tagged Raf-1 and different Pak3 and Src constructs. Lysates were generated from cells by the process described in Fig. 3. Phosphorylated Raf-1 species were immunoprecipitated with antisera specific for phospho-S338 (A) or phospho-S338/phospho-Y341 (B). The presence of FLAG epitope in the immunoprecipitates was measured by Western blotting and levels were normalized to the total expression of FLAG-tagged Raf-1, to give the relative levels of phosphorylation depicted in the graphs. Specificity of each antiserum was checked by the manufacturer and each was found not to have any significant cross-reactivity with inappropriate phosphopeptides.

not contribute to the substrate recognition of the site by either protein kinase. We conclude that the apparent cooperation between Pak and Src in Raf-1 activation in cells is not a consequence of enhancing the recognition site of either kinase by prior phosphorylation. Remaining possibilities include indirect enhancement of phosphorylation of S338 or Y341, or simply cooperating activation mechanisms.

3.5. Phosphorylation of Raf-1 by Pak3 and Src in cells

To further address this question, we examined the relative phosphorylation state of S338 in Raf-1 activated in COS-7 cells. Using phospho-specific antisera, Raf-1 phosphorylation in COS-7 cells was examined at S338, or S338/Y341 together. Expression of constitutive Pak^{ca} resulted in increased S338 phosphorylation (Fig. 4A). However, when Raf-1 was co-transfected with Pak^{ca} and Src^{ca}, a marked increase in S338 phosphorylation was observed above that seen with Pak^{ca} alone. This was also coincident with a large increase in dual phosphorylation of S338/Y341 (Fig. 4B). Increased dual S338/Y341 phosphorylation was also observed with expression of

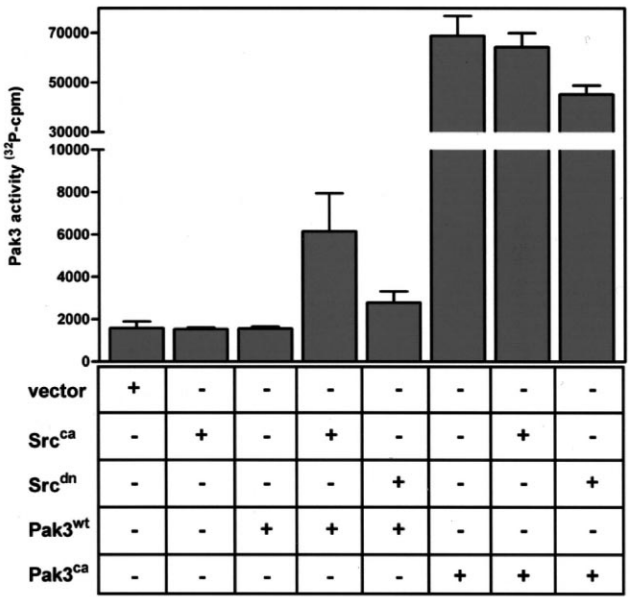

Fig. 5. Src stimulates Pak3 activity. HA-tagged Pak3 was immunoprecipitated from COS-7 cells transiently expressing the indicated Src and Pak3 constructs. The activity of the Pak3 in the immunoprecipitates was measured using the Raf-1 peptide substrate. Pak3 activity is expressed as γ-^{32}P incorporation (cpm). The data are representative of two independent experiments performed in quadruplicate.

Src^{ca} alone, however in this case S338 phosphorylation remained constant (Fig. 4B). Transfection of Pak^{dn} alone dramatically decreased S338 phosphorylation, as expected. Expression of Src^{dn} significantly decreased the basal level of phospho-S338 in Raf-1 but was less efficient at interfering with S338 phosphorylation by Pak^{ca}. We were unable to reproducibly measure phospho-Y341 levels. These data suggest that Src can promote Raf-1 phosphorylation on S338 by Pak in cells, possibly by increasing Pak activity or indirectly promoting access of the site to Pak for phosphorylation.

3.6. Src stimulates Pak3 activity in COS-7 cells

To observe the ability of Src to activate Pak in cells, Src^{ca} and Src^{dn} were co-expressed with wild type Pak3 and Pak3^{ca} in COS-7 cells. The HA-tagged Pak3 proteins were immunoprecipitated and assayed for relative activity using the Raf-1 peptide substrate (Fig. 5). The high constitutive activity was not altered by Src^{ca}, indicating that the activity of Pak3^{ca} was independent of Src^{ca} activity. However, Src^{dn} was able to attenuate Pak^{ca} activity, reducing the activity associated with the Pak3^{ca} immunoprecipitate by 36%. This finding would indicate that Src activity is able to modulate Pak3 activity. The failure of Src^{ca} to further increase Pak3^{ca} activity may reflect the possibility that, under the conditions of the experiment, Pak3^{ca} was already maximally activated. In contrast, Pak3^{wt} immunoprecipitates contained no significant activity, being no better than the vector or Src^{ca} alone controls. Most significant though, Src^{ca} dramatically stimulated Pak3^{wt} activity. In the absence of Src^{ca}, Pak3^{wt} activity was undetectable and equal to the vector control immunoprecipitate. Src^{ca}-stimulated Pak3^{wt} activity was approximately 10% of the activity measured in the Pak3^{ca} immunoprecipitates. This increase in Pak3^{wt} activity, however, was not due to differences in Pak3^{wt} expression since Pak3^{wt} levels were similar under both sets of conditions (data not shown). This observation, in conjunction with the observation that Src^{dn} is able to attenuate Pak3^{ca} activity, provides evidence that Src can modulate Pak3 activity in cells.

The small increase in Pak3^{wt} activity detected in the presence of Src^{dn} is likely an artifact and was equal to the background cpm obtained in immunoprecipitates from cells expressing Src^{dn} alone (data not shown).

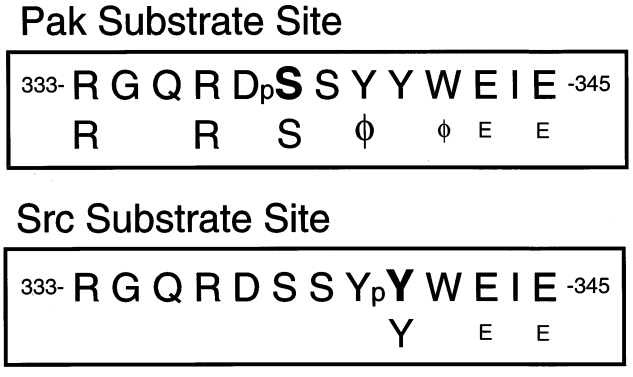

Fig. 6. Substrate sequence determinants for phosphorylation of Raf-1 on S338 by Pak3 and Y341 by Src. The linear sequence for Raf-1 residues 333–345 is displayed above the residues found to be most critical for phosphorylation of the sites designated pS and pY by recombinant Pak3 and immunoprecipitated Src. Smaller font size indicates only a moderate contribution for the designated residue in substrate recognition.

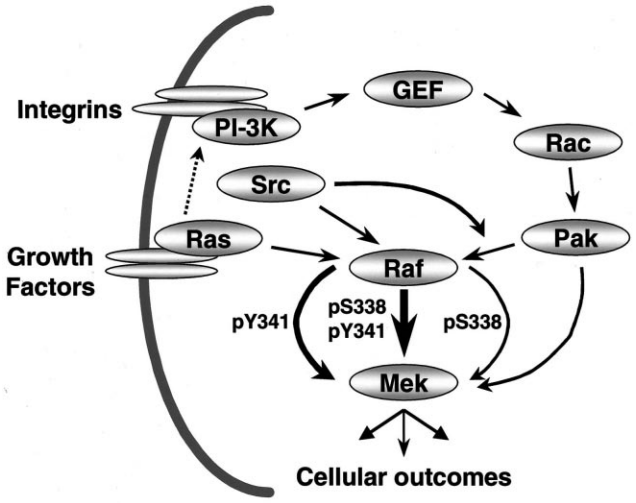

Fig. 7. Model showing the interplay between different receptor-activated pathways that signal through Raf-1. Precise biological outcomes are ultimately defined by the level of Raf-1 activation, which itself is represented by the weight of each arrow from Raf-1 to Mek. The degree of activation is a manifestation of the phosphorylation of Raf-1 in the S338-Y341 region of its CR3 domain.

4. Discussion

Regulation of Raf-1 has proven to be extremely complex and involves interaction with many regulatory and accessory proteins [1,3–6]. Since Raf-1 ultimately influences many cellular targets that control cell growth, division and differentiation [25–27], this kinase must be tightly regulated. The rela-

tionship between protein phosphorylation and activity regulation has been shown [28] and the multitude of post-translational modifications that are witnessed in Raf-1 regulation help provide this control [1]. We recently demonstrated S338 to be critical for Raf-1 activation in response to certain biological stimuli [9,10]. Pak was subsequently shown to phosphorylate S338 [13] as a consequence of signaling through phosphatidylinositol 3-kinase [14,15]. Here, we describe advances in understanding Raf-1 S338 and Y341 phosphorylation as well as cooperativity between Pak and Src in the regulation of Raf-1.

Substitution analysis of residues surrounding Raf-1 S338 showed that certain residues strongly influence Pak3-mediated phosphorylation (Fig. 6). Recognition of the S338 substrate sequence is strongly influenced by the presence of positively charged arginines at 333 (P−5 position) and 336 (P−2), an aromatic residue at 340 (P+2) and negatively charged glutamic acids at 343 (P+5) and 345 (P+7). This is in general agreement with other studies for Pak2 [17,20] and Pak1 [21], where arginines between P−2 and P−5 have been implicated in enhancing phosphorylation. The negative charges at P+5 and P+7 were only found to be important in context of the intact protein as a double glutamine substitution and may reflect a more gross structural effect. Loss of W342 (P+4) also reduced Pak phosphorylation of S338 in the intact CR3 protein but not in the peptide. In addition we learned that the presence of a negatively charged residue at P−1 does not influence substrate recognition by Pak while introduction of an acidic residue into the P+1 or P+2 positions is not tolerated. Substitution of S338 with threonine rendered CR3 a non-substrate for Pak3. This is in accord with the observation that a Raf-1 S338T mutant is inactive and fails to be phosphorylated in COS-7 cells (H.B. Diaz, unpublished result).

The sequence requirement for Src recognition of the Y341 site is less dependent on specific flanking residues than Pak recognition of S338 (Fig. 6). The presence of acidic residues at 343 (P+2) and 345 (P+4) enhanced phosphorylation of Y341. Substitution of charged and aromatic residues between the P−1 and P−8 positions had no significant effects on recognition of Y341 by Src. However, we did observe that the presence of acidic residues at 338 and 339 (P−3 and P−2) did enhance phosphorylation of Y341. This would be predicted based upon the biochemical observations of the dual regulation of Raf-1 by Pak and Src phosphorylation.

Two closely disposed phosphorylation sites in the Raf-1 catalytic domain have been the focus of much work [6,9,10, 12–15]. The critical nature of phosphorylation of S338 and Y341 is apparent, as a checkpoint for the progression of Raf-1 activation in response to many mitogenic stimuli. While S338 and Y341 reside in the Raf-1 CR3 domain, these residues are distant from the catalytic center. Their situation near the CR3 N-terminal, close to the glycine-rich nucleotide-binding P-loop, has been compared with similar residues in the transforming growth factor β type-1 receptor (TGFβR-1) [12]. In TGFβR-1, phosphorylation in this region is required for kinase activation. By comparison with B-Raf, Src and TGFβR-1, phosphorylation of S338 and Y341 is proposed to be structurally significant by allowing altered orientation of the C_α helix in kinase activation. Thus S338/Y341 may represent a Raf-1 'action' region, which only requires phosphorylation of one residue for progression of kinase activation. Thus, phosphorylation by Pak and Src may represent different methods of achieving the same conformational goal. Phosphorylation of S338 leads to low-level Raf-1 activation, while Y341 phosphorylation and S338/Y341 dual phosphorylation result in dramatically increased levels of Raf-1 activity. Structural reorganization permitted by phosphorylation might be more efficient with increased negative charge by dual phosphorylation of S338/Y341. This suggests the requirement of maximal phosphorylation for full Raf-1 activation, as seen in cell-based experiments. Based on our data set, we conclude that co-expression of constitutively active Src and Pak do cooperate to increase the degree of Raf-1 activation, likely due to the dramatic increase in S338 phosphorylation. The substrate specificity experiments demonstrate that prior phosphorylation of Y431 has little effect on the ability of Pak to phosphorylate Raf-1 on S338. However, Src clearly can increase Pak3 activity in cells. Whether this is a result of a direct activating phosphorylation on Pak3 or an indirect activation through Rac or Cdc42 is unclear. In addition, we observed that co-expression of constitutively activated Src and Pak3 dramatically increases phosphorylation of Raf-1 S338 even though Src is not observed to further increase the activity of constitutively activated Pak3. Possibly, activation of Src results in the phosphorylation and removal of a regulatory Raf-1 binding protein which obscures the S338 binding site. These conclusions are reinforced by the opposing effects observed with the dominant-negative alleles of Pak3 and Src. While the mechanism of action of the particular allele of Src^{dn} is believed to be substrate sequestration, results obtained with Pak^{dn} should be interpreted more loosely. This particular Pak3 allele functions to shut down Pak activation by binding non-productively to Rac and Cdc42 and certainly shuts down other Rac/Cdc42-controlled pathways as well. Unfortunately, we were unable to reliably measure tyrosine phosphorylation of Y341 to measure the reciprocal effects of Pak activity on Y341 phosphorylation in cells.

The cooperativity observed between Pak and Src in the regulation of Raf-1 suggests a hypothesis in which Pak-mediated S338 phosphorylation provides low-level Raf-1 activation, while Src-mediated Y341 phosphorylation induces high Raf-1 activation, in response to different stimuli. Dual phosphorylation of S338/Y341 would produce super-activation of Raf-1. Pak activation of Raf-1 was relatively small compared with that achieved by Src. Also, relative to Src alone, Pak3 only modestly increased the activation achieved by active Src. This alludes to Raf-1 regulation over a range of activities, which is significant since high activity promotes cell cycle arrest and senescence, whereas low Raf activity induces proliferation [25]. This is modeled in Fig. 7. The weight of arrows indicating phosphorylation of Raf-1 represents the level of Raf-1 activation achieved by each phosphorylation. The model shows the potential for Src family kinases to activate Raf-1, not only by direct Y341 phosphorylation, but also by direct regulation of Pak as well as by an additional undefined mechanism for regulating Pak access to Raf. Different degrees of Raf-1 activation are dependent upon the individual or combination of phosphorylation events mediated by these kinases. Even in the absence of exogenous active Pak, active Src may utilize endogenous active Pak in growing cells to achieve super-activation of Raf-1. Indeed, Mason et al. [12] propose that S338 phosphorylation may be the limiting factor in Src-mediated activation of Raf-1 and that, conversely, Y341 phosphorylation may be limiting in Ras-mediated Raf-1 activation.

The structural significance of S338/Y341 phosphorylation may provide a point at which signals from different pathways can feed into a common system, flowing through Raf-Mek-Erk. However, the co-operativity between Pak and Src, leading to super-activation, might represent another tier of Raf-1 regulation. While Pak or Src alone elicit lower individual levels of Raf-1 activation, further activation may be achieved in combination. This would provide a very powerful regulatory mechanism governing the precise level of Raf-1 activity. Since Raf-1 ultimately influences a vast array of downstream targets, different levels of Raf-1 activation might be manifested as very different biological outcomes.

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