Regulation of the Nonreceptor Tyrosine Kinase Brk by Autophosphorylation and by Autoinhibition*

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Brk (breast tumor kinase) is a nonreceptor tyrosine kinase that is most closely related to the Frk family of kinases, and more distantly to Src family kinases. Brk was originally identified in a screen for tyrosine kinases that are overexpressed in human metastatic breast tumors. To shed light on the activity and regulation of Brk and related tyrosine kinases, we expressed and purified Brk using the SF9/baculovirus system. We characterized the substrate specificity of Brk using synthetic peptides, and we show that the kinetic parameters $k_m$ and $k_{cat}$ both play a role in specificity. We carried out mass spectrometry experiments to show that Brk autophosphorylates within the predicted kinase activation loop and at additional sites in the N terminus. Autophosphorylation increases enzyme activity of wild-type Brk but not of a Y342A mutant form of Brk. We also carried out experiments to address the possible involvement of the Src homology (SH) 2 and SH3 domains of Brk in enzyme regulation. Mutation of a C-terminal tyrosine (Tyr-447) increases enzyme activity and SH2 domain accessibility, consistent with a role for this residue in autoinhibition. A proline-rich peptide activates Brk, suggesting that the SH3 domain is also involved in maintaining an inactive form of Brk. These biochemical results for Brk may aid in the understanding of other tyrosine kinases in the Frk family.

Brk is a nonreceptor tyrosine kinase that was identified in a study of kinase expression in human metastatic breast tumors (1). Brk expression was low or undetectable in normal mammary tissues or in benign lesions. However, approximately two-thirds of the breast tumors that were examined expressed significant levels of Brk, and 27% of the tumors overexpressed Brk by ≥5-fold (one tumor displayed a 43-fold increase in Brk expression) (2). Expression of Brk in cultured mammary epithelial cells makes them more sensitive to the mitogenic effects of EGF, and increases anchorage-independent proliferation (3). Brk expression can also partially transform NIH3T3 fibroblasts (3). These results suggest that inappropriate expression of Brk may contribute to the development of breast cancer.

Brk is usually considered to be a member of a separate family of cytoplasmic tyrosine kinases (the Frk family) that includes Frk, Brk, Srms, and Sik, the mouse ortholog of Brk (1, 4–6). Brk is more distantly related to the Src family kinases (there is 46% amino acid identity between Brk and c-Src) (1, 4). Unlike Src family kinases, Brk lacks an N-terminal consensus sequence for acylation and membrane association. Furthermore, the genomic structure of Brk is quite distinct from that of Src kinases, suggesting that Brk has diverged significantly from the Src kinases in evolution (7).

Brk possesses sequences that are predicted to form Src homology 3 (SH3) and Src homology 2 (SH2) domains (1). These domains bind to proline-rich sequences and to phosphotyrosine-containing sequences, respectively (8, 9). Three-dimensional structures of the Src family kinases Src and Hck (10–13) indicate that the SH3 and SH2 domains bind to other regions of the kinase in an intramolecular manner. The SH3 domain binds a polyproline type II helix in the linker region between the SH2 domain and the catalytic domain. The SH2 domain binds a sequence in the C-terminal tail that requires phosphorylation on Tyr-527 by c-Src kinase (Csk) (14). These intramolecular interactions stabilize an inactive (or “down-regulated”) conformation of the enzyme, and SH3 and SH2 ligands can potently activate Src kinases (15–18). In addition to the negative regulatory roles of the Src SH2 and SH3 domains, they are involved in specific substrate recognition (14, 19, 20). Binding of substrates by the SH2 and/or SH3 domains targets Src kinases to potential substrates and concomitantly activates the catalytic domain (21, 22).

There are several unanswered questions with regard to the structure, activity, and regulation of Brk. First, the substrate specificity of the Brk catalytic domain has not been characterized (two proteins, BKS and Sam68, have been identified to date as Brk substrates (Refs. 23 and 24)). Second, it is not clear whether the SH3 and SH2 domains of Brk function in an autoinhibitory manner. Brk possesses a tyrosine residue (Tyr-447) near the C terminus, but it is not known whether this site is phosphorylated or whether it is involved in Brk regulation. In Src family kinases, mutation of Tyr-527 (the analogous tyrosine) to Phe increases kinase activity, and expression of a Y527F mutant in fibroblasts leads to cell transformation (14). Mutation of Tyr-447 to Phe decreased the ability of Brk to induce anchorage-independent growth, suggesting that Brk might be regulated in a different manner than Src kinases (3). However, in contrast to these results, a Y447F mutant of Sik appeared to have increased tyrosine kinase activity when overexpressed in mammalian cells (24).

No purification of Brk kinase (or of any member of the Frk kinase family) has previously been carried out, and no structure-function studies have been reported to date. In this report, we have addressed several of these issues described above by mechanistic studies on the purified Brk protein.

4 The abbreviations used are: EGF, epidermal growth factor; Ni-NTA, nickel-nitrioltriacetic acid; HEK, human embryonic kidney; MALDI, matrix-assisted laser desorption ionization; SH, Src homology.

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**Materials and Methods**

**Brk Constructs and Mutagenesis**—The cDNA for Brk was the kind gift of Dr. Mark Crompton (School of Biological Sciences, Royal Holloway, University of London, London, United Kingdom). PCR was used to amplify the Brk coding sequence. The oligonucleotides used for PCR were: 5'-CCGGGATCCCTGGTAGCAGAGCTCACTC-GGGCCCATC and 5'-GCGGATCCCTTTTCAGCCTCGGAGCTGATGAGCCCTC. The Brk DNA was then subcloned as a BamHI/EcoRI fragment into plasmid pFastBac HTB (Invitrogen). Site-directed mutagenesis was performed using the Strategene QuickChange kit according to directions from the manufacturer. Mutations were confirmed by DNA sequencing.

**Protein Expression and Purification**—His-tagged wild-type Brk and mutants were expressed in Spodoptera frugiperda (Sf9) cells using the Bac-to-Bac baculovirus system (Invitrogen). For protein production, 0.6 liters of Sf9 cells were infected with high titer recombinant Brk baculovirus. After 3 days of infection, cells were harvested and washed with phosphate-buffered saline two times. Sf9 cells were lysed two times in a French pressure cell in 50 ml of buffer A (20 mM Tris, pH 8.5, 10% glycerol, 5 mM β-mercaptoethanol) containing protease inhibitors (5 mg/ml aprotinin, 5 mg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride), 0.1 mM vanadate, and 1 mM EDTA. Cell lysate was diluted to 200 ml, centrifuged, and filtered. The lysate was then mixed with 40 ml of Macro-Prep High Q resin (Bio-Rad) that had previously been equilibrated in buffer A. After rotating at 4°C for 1 h, the resin was washed with 100 ml of buffer A four times. Brk was then eluted from the High Q resin with 40 ml of buffer B (buffer A plus 1 mM NaCl). The protein was then loaded onto a 3-ml column of Ni-NTA resin (Qiagen) that had been pre-equilibrated in buffer A. The column was washed with three column volumes of washing buffer (buffer B plus 0.01 mM imidazole). Brk was eluted with buffer A containing 0.2 mM imidazole. One-mI fractions were collected and assayed for tyrosine kinase activity using the spectrophotometric assay (see below). Active fractions were pooled and stored at 4°C.

**Synthetic Peptides**—Peptides were prepared by solid phase synthesis on an Applied Biosystems automated model 431A peptide synthesizer. The peptides were purified by reverse-phase high pressure liquid chromatography and characterized by matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometry. The sequences of the peptides used are: Src-specific substrate, Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Ala-Lys-Lys-Lys-Gly (22, 25); Ab-specific substrate, Glu-Ala-Ile-Tyr-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Gly (25); EGF receptor-specific substrate, Ala-Glu-Glu-Glu-Tyr-Phe-Glu-Leu-Val-Ala-Lys-Lys-Lys-Gly (25); insulin receptor-specific substrate, Lys-Lys-Glu-Glu-Glu-Tyr-Met-Met-Met-Gly (28); SH2 binding peptide (pYEEI-resin), Glu-Pro-Gln-Tyr(P)-Glu-Ile-Pro-Ile (29); phospho-tyrosine peptides, Glu-Pro-Thr-Ser-Tyr(P)-Phe (29) and Thr. Phosphotyrosine was incorporated into peptides using N-Fmoc-O-phospho-t-tyrosine (where Fmoc is (N-9-fluorenylmethoxycarbonyl); Novabiochem).

**Kinase Assay**—Brk kinase activity was measured by a continuous spectrophotometric assay (22, 27). Reactions were performed at 30°C in 50 μl of buffer containing 20 mM Tris, pH 7.5, 3 mM MgCl₂, 1 mM phosphoenolpyruvate, 0.28 mM NADH, 89 units/ml pyruvate kinase, and 124 units/ml lactate dehydrogenase. For experiments with autophosphorylated Brk, the enzyme was preincubated with 0.5 mM ATP for 20 min at 30°C (22). Initial rates were measured in triplicate, and kinetic parameters were determined by fitting data to the Michaelis-Menten equation using nonlinear regression analysis. Vₐ₅₀ values for ATP were determined using a range of ATP concentrations (1–1000 μM) and 600 μM Src peptide substrate. Kᵥ₅₀ values for peptide were determined using a range of peptide concentrations (1–1000 μM) and 500 μM ATP. The activation constant, Kᵥ₅₀, was determined by nonlinear regression analysis of the activation curve as a function of ligand concentration using Equation 1.

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V_{act} = V_{max}[L]/K_{v50} + [L] \quad \text{(Eq. 1)}
\]

where \(V_{act}\) is velocity measured in the presence of ligand minus the velocity measured in its absence, \(V_{max}\) is the maximal activated velocity minus the velocity measured in the absence of ligand, and \([L]\) is the concentration of peptide (22). The \(K_v\) value was determined using enzymatic assay and varying concentrations of activator peptides (1–1000 μM). Kᵥ₅₀ determinations were performed using 500 μM ATP and 600 μM Src peptide substrate.

**Autophosphorylation Reactions**—Brk or Brk mutants (2 mg final concentration) were incubated with 0.5 mM (pYP) ATP (100–500cpm/ pmol) in buffer containing 20 mM Tris, pH 7.5, 5 mM MnCl₂, and 2 mM β-mercaptoethanol at 30°C for 20 min. In some experiments, Brk was preincubated with 0.5 mM unlabeled ATP in the same buffer for 30 min. Reactions were terminated by adding Laemmli buffer and analyzed by SDS-PAGE and autoradiography.

**MALDI Mass Spectrometry**—MALDI mass spectrometry was carried out at the Howard Hughes Medical Institute/Columbia University Protein Core Facility. 2 μg of Brk or autophosphorylated Brk was electrophoresed on SDS-PAGE and visualized by Coomassie staining. Brk bands were then excised from the gel; transferred to an acid-washed tube; rehydrated with water; crushed; washed three times for 20 min with 50 mM Tris, pH 8.0, 50% acetonitrile; dried; and incubated overnight at 35°C. The lyophilized sample was resuspended in 10 mg/ml 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile plus 0.1% trifluoroacetic acid containing angiotensin as an internal standard; and applied to a MALDI sample plate, which was dried and washed with water to remove excess buffer salts. MALDI mass spectrometric analysis was performed on a PerSeptive Voyager DE-RI mass spectrometer in the linear mode. The masses obtained in the MALDI mass spectrometry experiments were searched against predicted tryptic fragments of Brk using the program PeptideMass (28).

**Peptide Binding Experiments**—The pYEEI-resin was made by coupling the synthetic peptide Glu-Pro-Gln-Tyr(P)-Glu-Ile-Pro-Ile-Lys-Lys-Gly (0.5 ml of Affi-Gel 15 (Bio-Rad) in 0.1 M MOPS buffer, pH 7.5 (29). Prior to use in an experiment, the pYEEI-resin was washed extensively with 0.1 M MOPS. Binding reactions (final volume = 100 μl) were carried out by incubating 2 μg of Brk (or mutant Brk) with 30 μl of pYEEI-resin in buffer containing 50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 0.5 mM EDTA, 0.5 mM sodium vanadate, and 1 mM dithiothreitol. After a 30-min incubation at 4°C, the pYEEI-resin was washed with the same buffer. Brk bound to the immobilized pYEEI peptide was eluted in 30 μl of Laemmli buffer and resolved using SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane and detected with anti-Brk rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-rabbit horseradish peroxidase-conjugated secondary antibody, and an enhanced chemiluminescent (ECL) detection kit (Amer sham Biosciences).

**Expression in Human Embryonic Kidney (HEK) 293 Cells and Immunoprecipitation Experiments**—HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml streptomycin sulfate, and 100 μg/ml amphotericin B. Cells were plated 24 h before transfection. The Brk expression plasmid was derived from pRCCSV and was a gift from Dr. Mark Crompton. Cell transfection was performed using MIRUS TransIT (Mirus Corp.) according to the instructions from the manufacturer. The cells were incubated with 5 μg of plasmid for 8 h before changing to normal growth medium. The cells were harvested 48 h after transfection.

After harvesting and washing, 1 × 10⁵ 293 cells were lysed in ice-cold lysis buffer (25 mM Tris, pH 8.0, 2 mM EDTA, 1% Nonidet P-40, 140 mM NaCl) with protease inhibitors (5 mg/ml aprotinin, 5 mg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) for 30 min. The cell lysates were then centrifuged at 14,000 × g for 15 min at 4°C and precleared by incubation with 15 μl of protein A beads (Sigma) for 1 h at 4°C. For immunoprecipitation reactions, lysates were incubated with 1 μg of appropriate antibody and 15 μl of protein A beads (Sigma) overnight at 4°C. The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane. Western blotting was carried out using anti-Brk antibody or anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.). The immunoreactive proteins were visualized using horseradish peroxidase-conjugated secondary antibody and ECL.

**Results**

We produced a His-tagged version of full-length Brk (residues 1–451) using the Sf9/baculovirus expression system. For protein production, 0.6 liters of Sf9 cells were infected with high titer recombinant Brk baculovirus. Maximum Brk expression occurred after 3 days of infection. At this point, cells were harvested and lysed in an ice-cold buffer, and Brk was purified by successive chromatography on MacroPrep High Q anion exchange resin and a Ni-NTA column. The protein was greater than 95% pure as judged by SDS-PAGE with Coomassie staining, and it migrated at a position consistent with the expected 52-kDa molecular mass (Fig. 1).

The purified protein has a high specific activity, as measured
toward peptide substrates. Kinase assays were performed by two methods: (i) the phosphocellulose paper assay (30) and (ii) a coupled spectrophotometric assay (27). In the latter assay, the production of ADP is coupled to the oxidation of NADH, measured as a reduction in absorbance at 340 nm; this method has the advantage that it is a continuous assay. The two assays gave similar results, and the results shown in Table I are from the spectrophotometric assay. We first performed experiments with varying concentrations of ATP and at saturating amounts of peptide substrate to determine the $K_m$ for ATP. These experiments gave the following values: the $K_m$ for ATP = 83 μM and the $V_{max}$ = 37 nmol/min. We also found that Brk prefers manganese to magnesium as the divalent cation in the kinase reaction (data not shown).

The peptide/protein substrate specificity for Brk has not previously been investigated. We carried out experiments using four peptide substrates containing recognition motifs for different subfamilies of tyrosine kinases. Two of the peptides contain motifs that are preferred by nonreceptor tyrosine kinases (Src and Abl), and two peptides contain motifs that are preferred by receptor tyrosine kinases (EGF receptor and insulin receptor) (25). The results showed that the substrate specificity of Brk is more closely related to the specificity of Src and Abl. Of the substrates tested, the Src-specific synthetic peptide with the sequence Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Ala-Lys-Lys-Lys-Lys-Gly (22) had the lowest peptide with the sequence Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Leu-Val-Ala-Lys-Lys-Lys-Gly showed no significant phosphorylation by Brk, and the insulin receptor substrate, Lys-Lys-Glu-Glu-Glu-Tyr-Met-Met-Met-Met-Met-Gly, was phosphorylated with a $k_{cat}/K_m$ value ~6-fold lower than that of the Src peptide (Table I).

The conserved architecture of protein kinase catalytic domains consists of a smaller N-terminal lobe that contacts ATP, and a larger C-terminal lobe that makes most of the contacts with protein substrate (31). In many protein kinases, access to the active site is controlled by a flexible segment designated the activation loop (32). For example, in Src kinases, the activation loop containing unphosphorylated Tyr-416 is positioned such that access of peptide substrates to the catalytic machinery is blocked (12). Autophosphorylation of Tyr-416 increases the catalytic activity of the enzyme by causing the activation loop to move out of the substrate-binding site, thereby allowing access to substrates. Brk contains a tyrosine (Tyr-342) at a position within the kinase domain that could serve a similar function.

### Table I

| Peptide                      | $V_{max}$ | $K_m$ | $k_{cat}/K_m$ |
|------------------------------|-----------|-------|---------------|
| Src substrate                | 37 ± 5    | 105 ± 12 | 1.8 ± 10^4    |
| Insulin receptor substrate   | 26 ± 1.6  | 540 ± 103 | 2.5 ± 10^5    |
| Abl substrate                | 292 ± 21  | 655 ± 84 | 2.3 ± 10^4    |
| EGF receptor substrate       | No phosphorylation* |

*The EGF receptor gave no detectable phosphorylation in the spectrophotometric assay, indicating that the $k_{cat}/K_m$ for this substrate is ≤50 nmol/min. 

We carried out experiments to test whether Brk is activated by autophosphorylation within the activation loop. First, we incubated Brk with [$\gamma$-32P]ATP in kinase reaction buffer (see “Materials and Methods”) for 20 min at 30 °C. As indicated, the proteins were preincubated with or without 500 μM unlabeled ATP in the same buffer for 30 min. Reactions were terminated by adding Laemmli buffer and were analyzed by SDS-PAGE and autoradiography.

We carried out mass spectrometry experiments to identify autophosphorylation sites on Brk. We analyzed two samples: (i) Brk directly purified from baculovirus-infected SF9 cells; and (ii) Brk after an extended autophosphorylation reaction. The two samples were electrophoresed on SDS-PAGE, and the protein bands were reduced and alkylated with iodoacetamide, digested with trypsin, and analyzed by MALDI mass spectrometry (Fig. 3). We focused on tryptic fragments of Brk that were found in the unphosphorylated state and that were shifted by the mass of a phosphate (+80 daltons) in the autophosphorylated sample. The major peak fitting these criteria (labeled a in Fig. 3) has a mass that corresponds to a peptide from the putative activation loop of Brk. This tryptic peptide contains two tyrosines (Tyr-342 and Tyr-351). Two other peaks (b and c in Fig. 2) correspond to alternate trypsin cleavage fragments containing Tyr-342 and Tyr-351. The sequence surrounding...
FIG. 3. Identification of Brk autophosphorylation sites by MALDI mass spectrometry. Brk directly from SF9 cells or autophosphorylated Brk (2 μg) was electrophoresed on SDS-PAGE and visualized by Coomassie staining. The Brk bands were excised from the gel and subjected to in-gel trypsin digestion. Top spectrum, tryptic fragments of native Brk (no autophosphorylation) analyzed by MALDI mass spectrometry. Lower spectrum, tryptic fragments of autophosphorylated Brk analyzed by MALDI mass spectrometry. Fragments a–f are Brk peptides that are present in their unphosphorylated state in the Brk sample and are present in their phosphorylated state (a'–f', + 80 daltons) in the autophosphorylated sample. Except for a small amount of peak b', none of the phosphorylated peptides was observed in the unphosphorylated sample. The autophosphorylated sample contained unphosphorylated peptides c and e as well as the phosphorylated peptides. The masses, Brk sequences, and phosphorylation sites are summarized at the bottom of the figure.
Tyr-342 is well conserved between Brk and the major autophosphorylation site in Src (Tyr-416). Tyr-351 is not conserved in Src (the corresponding residue is an Ile). Thus, Tyr-342 is likely to be the major site of autophosphorylation in Brk, although we cannot exclude the involvement of Tyr-351. We also observed three additional tryptic fragments (d, e, and f) that showed the +80 shift in mass upon autophosphorylation, although the peaks of phosphorylated peptides were smaller in these cases. These tryptic fragments are derived from the N-terminal region of Brk. We observed remaining unphosphorylated peptides c and e in the autophosphorylated sample, indicating substoichiometric phosphorylation. Other unphosphorylated peptides were not present in detectable amounts in the autophosphorylated sample. We did not observe any evidence for phosphorylation at Tyr-447 in the C terminus of Brk.

To assess the importance of the putative autophosphorylation site at Tyr-342, we produced a mutant form of Brk in which Tyr-342 was changed to Ala. This mutant was expressed in Sf9 cells and purified by the same strategy used for wild-type Brk. We also expressed and purified two other Brk mutants: a Y447F mutant in the putative C-terminal regulatory site, and a double mutant containing substitutions at both the Tyr-342 and Tyr-447 sites. We first analyzed the autophosphorylation activity of the mutants (Fig. 2). The Y342A and Y342A/Y447F mutants showed decreased, but still significant, autophosphorylation when incubated with [γ-32P]ATP and analyzed by autoradiography. The autophosphorylation activity of the Y447F mutant was comparable with that of wild-type Brk (Fig. 2). In each case, preincubation of the enzyme with manganese and unlabeled ATP reduced the level of phosphate incorporation.

The catalytic activity of most protein kinases is controlled by phosphorylation within the activation loop. We analyzed the effect of autophosphorylation on enzymatic activity for wild-type Brk and for the mutants. In these experiments, autophosphorylation of wild-type Brk caused a ~2-fold increase in enzyme activity, as measured toward a synthetic peptide substrate (Fig. 4). The Y342A mutant form of Brk exhibited a 3-fold lower specific activity than wild-type Brk, consistent with a role for this residue in kinase activation (Fig. 4). Preincubation of the Y342A mutant with ATP and MnCl2 resulted in no increase in kinase activity (Fig. 4). The Y447F mutant form of Brk had a ~2.5-fold higher specific activity than wild-type Brk in this assay, suggesting that Tyr-447 may in fact play a role in Brk autoinhibition. Autophosphorylation of the Y447F mutant did not increase the enzymatic activity. Finally, the Y342A/Y447F double mutant displayed a level of activity that was intermediate between wild-type Brk and the Y342A mutant. As in the case of the other mutants, autophosphorylation of the double mutant did not appear to increase enzyme activity (Fig. 4). Thus, these experiments provided preliminary evidence that Brk activity is increased by autophosphorylation at Tyr-447.

In the down-regulated form of Src kinases, intramolecular binding of the SH2 and SH3 domains inhibits enzyme activity (33). Disruption of either of these interactions promotes autophosphorylation in the activation loop and enzyme activation (15–18). We have shown previously that micromolar concentrations of synthetic peptide ligands for the SH3 and SH2 domains of Src kinases activate the enzymes, as measured by the spectrophotometric assay (15, 22). Because the Y447F mutant form of Brk displayed an increased kinase activity, we tested for the ability of synthetic SH3 and SH2 ligands to activate Brk and Y447F Brk. We used two SH2 ligands: (i) a phosphotyrosine-containing peptide that contains the consensus sequence for binding to the Src SH2 domain, pYEEI (22); and (ii) a phosphotyrosine-containing peptide that corresponds to the C-terminal sequence of Brk (PTSpYENPT). We included the latter peptide in the study because the consensus sequence for binding to the Brk SH2 domain is unknown, and we reasoned that the C terminus of Brk itself might provide a high affinity ligand for the SH2 domain. However, we were unable to detect any increased Brk activity in the presence of micromolar concentrations of these Tyr(P)-containing peptides (data not shown). In the presence of high (1 mM) concentrations, we observed a modest ~1.4-fold increase in kinase activity (Fig. 5).

We also tested a proline-rich peptide that binds to the SH3 domains of Src family kinases (34) as a potential activator of Brk. This peptide was able to activate wild-type Brk with a $K_{act}$ (concentration needed for half-maximal activation) of 134 μM (Fig. 6). This is comparable with the $K_{act}$ of 107 μM measured for this peptide as an activator of Src kinases (34). Thus, it is likely that the SH3 domain of Brk plays a role in enzyme regulation. We carried out similar experiments with the Y447F mutant of Brk. The proline-rich peptide gave a small amount of additional activation in the Y447F mutant of Brk (Fig. 6).

As another test for intramolecular interactions involving the SH2 domain of Brk, we measured the accessibility of the ligand binding surface of the SH2 domain. In these experiments, we compared the ability of wild-type Brk, Y342A Brk, Y447F Brk, and Y342A/Y447F Brk to bind to an immobilized ligand for the SH2 domain (a pYEEI-containing peptide). We showed previously that C-terminally phosphorylated Src kinases do not bind appreciably to immobilized pYEEI peptide under the conditions of this assay, whereas Src kinases dephosphorylated at Tyr-527 bind well (29). Wild-type Brk bound weakly to the immobilized SH2 ligand (Fig. 7, lane 2). The Y342A mutation...
alone produced a small increase in binding (Fig. 7, lane 3). There was a significant increase in binding for the Y447F and Y342A/Y447F mutants (lanes 4 and 5, respectively), suggesting that elimination of the C-terminal tyrosine residue increases the availability of the SH2 domain.

We next tested for Brk autoinhibition in transfected HEK 293 cells. We introduced the following forms of Brk into 293 cells: wild-type Brk, Y342A, Y447F, and W44A. Trp-44 is a conserved residue in the SH3 domain, and the W44A mutation is predicted to disrupt interactions with polyproline sequences (23). As a negative control, we also transfected 293 cells with K219M Brk (a kinase-inactive mutant, containing a substitution at the ATP-binding site). All of the mutants were expressed at levels comparable with wild-type Brk (Fig. 8, middle panel). After transient expression, we analyzed cellular proteins for phosphotyrosine content as a measure of in vivo Brk activity (Fig. 8, top panel). The results show that the Y447F and W44A mutants have higher kinase activity than wild-type Brk, with Y447F showing the highest level of activity. To test for autophosphorylation, Brk was immunoprecipitated from 293 cell lysates and analyzed by SDS-PAGE with anti-phosphotyrosine Western blotting. As expected, the K219M mutant showed no autophosphorylation (Fig. 8, bottom panel). The
Y342A mutant showed greatly reduced autophosphorylation, although a small level of autophosphorylation remained, consistent with our earlier observations on purified Brk (Fig. 2). The Y447F and W44A mutants displayed a higher autophosphorylation activity than wild-type Brk; Y447F gave the highest level of activity (Fig. 8, bottom). These results suggest that the Y447F and W44A mutations disrupt the normal intramolecular regulation of Brk and increase the catalytic activity of Brk.

DISCUSSION

Using recombinant Brk purified from insect cells, we have carried out experiments to investigate the activity, substrate specificity, and regulation of this enzyme. We studied peptide substrate specificity of the Brk catalytic domain using peptides that are specific for four representative tyrosine kinases (Src, Abl, EGF receptor, and insulin receptor). The two peptides specific for nonreceptor tyrosine kinases were superior to peptides specific for receptor kinases (Table I). The Src-specific peptide had the lowest \( K_m \) of the peptides tested. However, the best peptide (in terms of \( k_{cat}/K_m \)), the apparent second-order rate constant that is an overall measure of catalytic efficiency) was the Abl-specific peptide, containing the Ile-Tyr-Ala-Ala-Pro-Phe recognition motif (25). Efficient phosphorylation of this peptide was driven primarily by a dramatically higher \( V_{max} \) compared with the other peptides tested. The results suggest that, in addition to \( K_m \) effects, the \( k_{cat} \) parameter plays a role in the discrimination of specific from nonspecific substrates by Brk. The results also indicate that superior peptides might be created that combine the optimal \( K_m \) effect of the Src peptide and the higher \( k_{cat} \) of the Abl sequence.

The synthetic peptide studies described in Table I focus on the specificity of the Brk catalytic domain. However, in vivo protein recognition is likely to depend also on interactions with other noncatalytic regions of Brk. For Src kinases, many cases have been described in which the enzymes are recruited to cellular substrates by SH3 and/or SH2 domain interactions (14, 35). The polyproline or phosphotyrosine motifs in such substrates activate the Src kinases by relieving autoinhibitory interactions, and tether the substrate to the enzyme, facilitating phosphorylation. The available evidence suggests that Brk relies on noncatalytic regions for substrate recognition as well. A mutant form of Brk with a defective SH3 domain displays reduced phosphorylation of the adaptor-like protein BKS (23). Mutations in the SH2 domain of Brk lead to reduced complex formation with BKS and a somewhat lower level of BKS phosphorylation. The most dramatic reduction in BKS phosphorylation, however, was observed in experiments with BKS possessing a mutation in its SH2-like sequence. This result suggests that the SH2-like sequence of BKS binds to another phosphotyrosine on Brk; our mass spectrometry studies (Fig. 3) suggest that tyrosines in the activation loop or in the N terminus of Brk might be possible sites for interaction. Sik, the mouse ortholog of Brk, also depends on SH3/SH2 domain interactions for maximal substrate phosphorylation of the RNA binding protein Sam68 (24). Furthermore, the proline-rich P3 region of Sam68 was shown to be the primary determinant for binding to the SH3 domain of Brk or Sik.

We obtained evidence that Brk is regulated by autophosphorylation. First, in the presence of manganese and \( ^{32}P \)-labeled ATP, the enzyme incorporates radiolabeled phosphate (Fig. 2). Second, our mass spectrometry experiments (Fig. 3) demonstrate specific incorporation of phosphate into the activation loop, most likely at Tyr-342, the site homologous to the Tyr-416 autophosphorylation in Src. Brk also appears to autophosphorylate additional sites in the N terminus at lower levels. The significance of these phosphorylations is not understood at present, but they may account for the remaining phosphorylation observed when the Y342A mutant was incubated with \( ^{32}P \)-ATP (Fig. 2). Third, when expressed in HEK 293 cells, the Y342A mutant form of Brk showed reduced autophosphorylation (Fig. 8). Finally, we observed a small but significant increase in the activity of Brk after pre-incubation with manganese and ATP (Fig. 4). The ~2-fold change in activity observed for Brk stands in contrast to Src-family kinases, where an increase of >10-fold is observed upon autophosphorylation at Tyr-416 using the same assay system (22). Not all protein kinases are activated to the same extent by autophosphorylation, because of differences in intramolecular inhibition of the ATP and peptide binding sites by the activation loop (32). We considered the possibility that the Brk purified from SF9 cells was already substantially phosphorylated on Tyr-342, so that additional phosphorylation would not produce a large change in activity. To address this possibility, we treated Brk with the tyrosine phosphatase YOP and then re-purified the enzyme. The dephosphorylated form of Brk showed the same small increase in enzyme activity upon autophosphorylation (data not shown).

Previous studies on Brk expressed in mammalian cells have not unambiguously shown whether the enzyme is regulated by autoinhibitory interactions. In particular, a Y447F mutant of Brk had a decreased ability to induce anchorage-independent growth (3), whereas a Y447F mutant of Sik had increased tyrosine kinase activity when overexpressed in mammalian cells (24). Our results on Brk provide three pieces of evidence that Tyr-447 is involved in enzyme regulation: (i) The Y447F mutant of Brk had increased specific activity (relative to wild-type Brk), as measured toward synthetic peptide substrates (Fig. 4); (ii) the Y447F mutant showed an increased capacity to bind to an immobilized ligand for the SH2 domain, suggesting that an inhibitory interaction was disrupted in the mutant (Fig. 7); and (iii) when expressed in HEK 293 cells, the Y447F mutant form of Brk showed increased in vivo tyrosine kinase activity and a higher level of autophosphorylation. We also attempted to activate Brk by addition of SH2 domain ligands, but only modest activation was observed, and only at very high concentrations of ligand (Fig. 5). The ligands used in this experiment were a peptide specific for the Src SH2 domain (containing pYEEI) and a phosphopeptide mimicking the Brk C-terminal tail. It is likely that the Brk SH2 domain has a different specificity from that ofSrc (24, 36), and such a ligand might be more effective in activating Brk. Our results therefore point to a role for Tyr-447 in Brk regulation, and are consistent with the prior results on expression of Y447F Sik (24). We speculate that the decrease in transformation activity seen previously for Y447F Brk (3) could be the result of an interference with intermolecular interactions between Brk and other cellular proteins that are important for transformation.

We obtained evidence that the SH3 domain of Brk also plays a role in enzyme regulation (Fig. 6). A Pro-rich peptide activates Brk with a potency that is similar to that observed for Src kinases, where the importance of the SH3 domain in enzyme regulation has been well documented. The W44A mutant form of Brk also showed higher kinase activity than wild-type Brk when expressed in mammalian cells (Fig. 8).

One question that is not resolved in our studies is whether the Brk we produced in insect cells represents the completely down-regulated form of the enzyme. For studies on Src and Hck, it was necessary to treat the enzymes with Csk to generate the C-terminally phosphorylated, down-regulated forms of the enzymes. The identity of the kinase responsible for C-terminal phosphorylation of Brk is unknown; we were unable to phosphorylate Brk by treatment with recombinant Csk. We did not detect...
any phosphorylation of Tyr-447 by mass spectrometry in either the unphosphorylated or the autophosphorylated sample of Brk. It is likely that the autoinhibitory interactions will be more pronounced when conditions are discovered for production of the homogeneous down-regulated form of Brk.

The studies described here represent the first attempt to characterize the activity and regulation of Brk using purified enzyme. Because no other Frk family nonreceptor tyrosine kinase has yet been studied in detail, the results may also shed light on the structure and biochemical features of other enzymes of this class.

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