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Structure–activity relationship of a novel peptide substrate for p60$^{c\text{-}src}$ protein tyrosine kinase

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SUMMARY

We recently reported the identification of a peptide (YIYGSFK) as an efficient substrate for p60$^{c\text{-}src}$ using a random combinatorial peptide library screening method. Over 70 analogues of YIYGSFK were designed and synthesized on beads and their phosphorylation on solid phase by p60$^{c\text{-}src}$ was quantitated by the PhosphorImager. A hydrophobic L-amino acid in position 2 and a basic amino acid in position 7 proved crucial for activity as a substrate. In addition, the L-tyrosine residue at position 3 was critical as the phosphorylation site and was found to be stereospecific, as substitution with the D-enantiomer at this position rendered the peptide totally inactive.

INTRODUCTION

c-*src*, the normal cellular homologue of the Rous sarcoma virus oncogene (v-*src*), encodes the p60$^{c\text{-}src}$ protein tyrosine kinase (PTK) that catalyzes the phosphorylation of proteins on tyrosine residues [1,2]. p60$^{c\text{-}src}$ is found in most normal tissues in humans. With the exception of platelets [3] and neurons [4], its level and activity are rather low. However, its activity is markedly increased in several human tumors [5–7]. One of the major thrusts of PTK research is to identify and characterize the physiological substrates for these enzymes [8].

In the last few years, random combinatorial library methodologies have proven to be valuable tools for identifying ligands of macromolecular targets such as receptors and for discovering

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Abbreviations: β, β-alanine; ε, ε-aminocaproic acid; Ac, *N*-acetyl; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; BSA, bovine serum albumin; Cha, L-cyclohexylalanine; Chg, L-cyclohexylglycine; Dab, L-diaminobutyric acid; Dap, L-diaminoproprionic acid; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HOBt, *N*-hydroxybenzotriazole; MeF, *N*-methyl-L-phenylalanine; MeG, *N*-methylglycine; MeI, *N*-methyl-L-isoleucine; MES, 2-[*N*-morpholino]ethanesulfonic acid; Nle, L-norleucine; Orn, L-ornithine; TFA, trifluoroacetic acid; Z-Sar, benzyloxycarbonyl-sarcosine; Z-Tyr, benzyloxycarbonyl-L-tyrosine.

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potential new drugs [9–13]. One method is based on the 'one-bead, one-peptide' concept, in which millions of different peptide beads can be synthesized. Each bead displays only one peptide [14,15]. Using this method, we identified peptide ligands for a variety of targets, including monoclonal antibodies [14,16,17], MHC class I molecules [18], streptavidin [14,19], avidin [19], gpIIb-IIIa integrin [20] and even small organic molecules, such as indigo dye [21]. Recently we modified this technique to elucidate posttranslational modification sites of proteins [22,23]. [γ-^{32}P]ATP and cAMP-dependent protein kinase were added to a random peptide-bead library. The peptides with the correct substrate motifs were radiolabelled, and the [^{32}P]-labelled beads were isolated for microsequencing. Several peptides with the RRXS motif were isolated [23], and this motif correlated very well with that reported in the literature [24,25], thus validating the methodology. We recently extended this technology to identify the peptide substrates for p60$^{c\text{-}src}$ PTK [26]. After screening a limited heptapeptide library (500 000 beads), a peptide with the sequence YIYGSFK was identified. Using a ferric chelation column chromatography approach, Songyang et al. recently reported the screening of a 15-mer 'oriented' peptide library (MAXXXXYXXXXAKKK, where X = all 20 eukaryotic amino acids except tryptophan, cysteine, tyrosine, serine and threonine) with a variety of PTKs (including c-Src), and determined the substrate motif of c-Src as EEIYGEFF [27]. In this paper, we report on the design and synthesis of more than 70 analogues of YIYGSFK and their structure–activity relationships (SARs) for Src kinase specificity.

MATERIALS AND METHODS

Human p60$^{c\text{-}src}$ was purchased from UBI (Lake Placid, NY), [γ-^{32}P]ATP was obtained from ICN Biomedicals Inc. (Irvine, CA), BSA (fraction V), MES, magnesium chloride, phenol, anisole, and ethanedithiol were obtained from Sigma (St. Louis, MO), Fmoc-protected amino acids, TFA, piperidine, BOP, HOBt, and DIEA were purchased from Advanced ChemTech (Louisville, KY), TentaGel S resin was obtained from Rapp Polymere (Tübingen, Germany) and DMF and methanol were purchased from Baxter (McGaw Park, IL).

Solid-phase peptide synthesis

Solid-phase peptides were synthesized with Fmoc chemistry [28,29], which was performed manually in a reaction vessel made from polypropylene columns with a polyethylene frit (2.2 × 8.5 cm) from Pierce Chemicals (Rockford, IL). The TentaGel S resin already has a hydrophilic polyethylene glycol linker and a free amine, and is suitable for coupling. The Fmoc-protected amino acids and the coupling reagents BOP, HOBt, and DIEA were added to the TentaGel S resin in threefold molar excess. The average coupling time at room temperature was 1 h. Completion of coupling was confirmed with the ninhydrin test. The Fmoc group was deprotected with 20% piperidine (v/v) in DMF (one treatment for 5 min, followed by another one for 15 min). The side-chain protecting groups were removed by using a mixture of TFA/phenol/ethanedithiol/anisole (94:2:2:2, v/w/v/v). The beads were then neutralized with 10% DIEA in DMF, washed with DMF, methanol and doubly distilled water, and stored in PBS with 0.05% gelatin and 0.05% sodium azide at 4 °C.

Peptide phosphorylation on solid phase

About 100 peptide-beads from each sample were washed extensively (6×) with MES buffer (30 mM MES, 10 mM magnesium chloride, 0.4 mg/ml BSA, pH 6.8). The phosphorylation reaction was performed in 20 µl MES buffer (pH 6.8) containing 1.5 units human p60$^{c\text{-}src}$, 0.1 µM [γ-^{32}P]ATP (specific activity 25 Ci/mmole) and about 100 peptide-beads. After 2 h of incubation at room temperature under gentle shaking, the beads were washed 6× with PBS-Tween (0.68 M NaCl, 10 M KCl, 40 mM Na_2HPO_4, 7 mM KH_2PO_4, 0.05% Tween 20, pH 1).

Quantitation of peptide phosphorylation on solid phase by the PhosphorImager

About 20 thoroughly washed [^{32}P]-labelled beads were suspended in 1 ml 1% agarose solution (w/v) at 80–85 °C, carefully poured onto a section of a glass plate that had been divided into 2×4 cm sections with a wax pencil, and air dried overnight. The immobilized beads were then exposed to the storage phosphor screen for 12 h at room temperature. The exposed screen was read by the 425S PhosphorImager (Molecular Dynamics (Sunnyvale, CA)) and relative phosphorylation of the peptide-beads was quantitated. In order to compare the relative phosphorylation of each peptide from different experiments, control YIYGSFK-beads were included in each experiment. The relative phosphorylation of each peptide was calculated from the following equation:

$$\frac{\text{total phosphorylation of peptide-beads / number of peptide-beads quantitated}}{\text{total phosphorylation of YIYGSFK-beads / number of YIYGSFK-beads quantitated}} \times 100\%$$

RESULTS

Figure 1A shows a representative 'PhosphorImage' of the time course of phosphorylation of YIYGSFK on the solid phase. The YIYGSFK-beads were incubated with [γ-^{32}P]ATP and p60$^{c\text{-}src}$ PTK. The reaction was terminated with an equal volume of 50% acetic acid at various times. Panels a, b, and c in Fig. 1A represent triplicate experiments. The PhosphorImage demonstrates that the signal intensity was time-dependent. Figure 1B shows the quantitative analysis of phosphorylation of YIYGSFK on the solid phase by the PhosphorImager, expressed in pixels/bead. Under the conditions studied, phosphorylation rates of YIYGSFK were constant for more than 120 min. We arbitrarily assigned the quantitation of YIYGSFK phosphorylation on the solid phase at 120 min a value of 100%. In all subsequent experiments, YIYGSFK-beads were included as an internal standard. In some experiments, occasional strongly labelled spots were detected in a background of weakly or unlabelled beads (see the two arrows in Fig. 1A). These strongly labelled spots were artifacts due to [^{32}P]-impregnated contaminated particulate matter or large

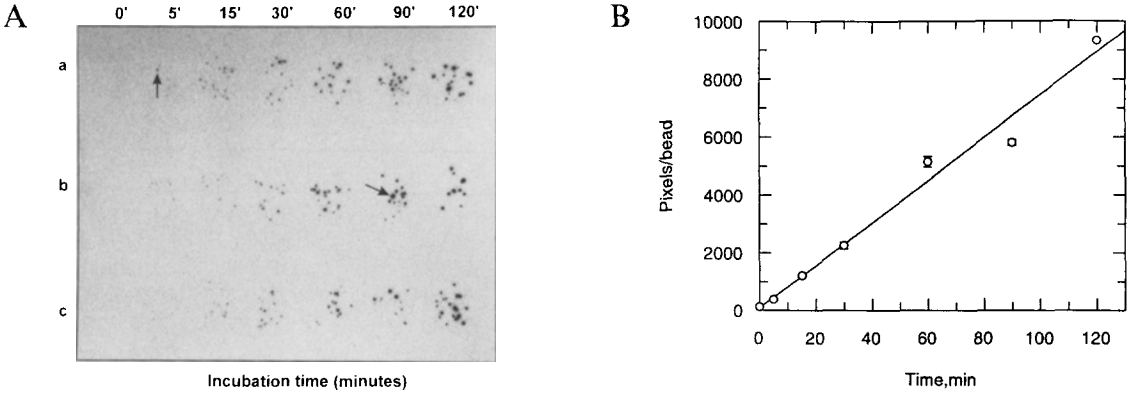

Fig. 1. (A) Representative 'PhosphorImages' of the time course of phosphorylation of YIYGSFK on solid phase by p60$^{c\text{-}src}$ PTK. The reaction mixture without the enzyme was considered at 0 min. (a), (b), and (c) are triplicate experiments. The two arrows point to the artifacts as a result of [^{32}P]-impregnated contaminated particulate matters or large bead aggregates, which were excluded from the quantitative analysis. See the text for an explanation. (B) Quantitative analysis of the time course of phosphorylation of YIYGSFK-beads with p60$^{c\text{-}src}$ using the PhosphorImager, expressed in pixels/bead. About 20 YIYGSFK-beads were quantitated in each of the time points. Triplicate experiments were performed, with error bars indicating the standard deviation of the mean. The concentration of [γ-^{32}P]ATP was 0.1 μM.

TABLE 1
PHOSPHORYLATION SCALE OF YIYGSFK ANALOGUES BY $p60^{c\text{-}src}$ ON SOLID PHASE: DELETION ANALOGUES

Peptide	Phosphorylation scale[a]
YIYGSFK	100
IYGSFK	36
YGSFK	9
AcYIYGSFK	8
AcIYGSFK	9
AcYGSFK	3
YIYGSF	7
YIYGS	2
YIYG	2
YIY	3
YIYββFK	23
YIYβFK	16
YIYεFK	22
YIYPFK	2

[a] Phosphorylation was quantitated by PhosphorImager. YIYGSFK = 100%.

bead aggregates, which could easily be visualized under the dissecting stereomicroscope. These artifactual spots were excluded from the quantitative analysis.

Tables 1–3 summarize the SAR data obtained for YIYGSFK analogues. Deletion analogues were synthesized and tested to determine the minimal sequence required for activity (Table 1). When Tyr^1 was deleted, only 36% phosphorylation remained. When Tyr^1 and Ile^2 were both deleted, only 9% phosphorylation was detected. N-acetylation of IYGSFK and YGSFK did not restore the activity. Any deletion from the carboxyl terminus rendered the peptide inactive. When Gly^4-Ser^5 were substituted by β-alanine-β-alanine, β-alanine, or ε-aminocaproic acid, only 23%, 16% and 22% phosphorylation was detected, respectively. Replacing Gly^4-Ser^5 with proline was not tolerable. N-acetylation of the parent compound YIYGSFK resulted in a dramatic decrease in phosphorylation.

Table 2 and Table 3 summarize data obtained from analogues with single L-amino acid or D-amino acid substitution, respectively. Tyr^1 appeared to be critical for full activity. However, 65% activity still remained when L-Tyr was substituted by D-Tyr, suggesting that stereospecificity was not crucial at this position. When Phe, Thr or Trp were used in position 1, 76%, 71%, and 41% activity were still retained. This suggests that Tyr^1 is not the phosphorylation site. Replacing Tyr^1 with Z-Tyr or Z-Sar resulted in 90% loss of activity. When Tyr^1 was replaced by hydrophobic (Ala, Cha), polar (Ser) or basic (Dap, Dab, Orn) amino acids, 8–30% activity remained.

For position 2, a hydrophobic amino acid was

TABLE 2
PHOSPHORYLATION SCALE OF YIYGSFK ANALOGUES BY $p60^{c\text{-}src}$ ON SOLID PHASE: SINGLE L-AMINO ACID SUBSTITUTION

Y	I	Y	G	S	F	K
Y = 100	I = 100	Y = 100	G = 100	S = 100	Y = 175	K = 100
F = 76	L = 92	F = 7	S = 36	T = 92	F = 100	Dab = 86
T = 71	V = 90	A = 4	A = 34	A = 89	MeF = 30	Orn = 82
W = 41	Chg = 29		MeG = 12	V = 48	W = 25	Dap = 77
S = 30	A = 21		K = 7	G = 40	Cha = 22	R = 26
E = 27	Nle = 12		N = 6	Y = 34	A = 11	A = 10
Cha = 23	K = 7		L = 1	K = 33	H = 7	H = 5
A = 16	E = 6		D = 0	N = 30		D = 1
Orn = 15				D = 1		
Dap = 13						
Z-Sar = 10						
Z-Tyr = 10						
Dab = 8						

found to be critical for activity. When Ile was replaced by Val and Leu, 90–92% activity remained. However, only 12% activity was retained when Nle was used instead. Replacement with Chg or Ala resulted in 71% or 79% reduction in activity. Less than 10% activity was observed when Lys or Glu were used. When Ile^2 was replaced by its D-enantiomer, there was a 87% reduction in activity.

Tyr^3 was essential for activity. Substitution with Ala, Phe or D-Tyr at this position almost completely abolished activity, suggesting that Tyr^3 is the phosphorylation site.

Gly^4 appeared to be extremely important. Substitution with Ala and Ser resulted in about 66% and 64% reduction of activity. Interestingly, the activity decreased dramatically to 2% and 12% when D-Ala and N-methylated Gly were used. Replacement with hydrophobic (Leu), acidic (Asp), basic (Lys), or polar (Asn) residues was completely intolerable, as less than 7% activity remained.

Ser^5 is important but could be substituted by Thr or Ala, as replacement with these residues resulted in only about 10% loss of activity, and substitution by Val resulted in a 52% reduction in activity. However, about 60–70% activity was lost when Ser was replaced by Asn, Lys, Tyr, or Gly. Surprisingly, only about 10% activity was retained when D-Ala, D-Thr, or D-Ser were used. Replacement with Asp completely abolished the activity.

An aromatic residue at position 6 appeared to be crucial. Although 22–46% activity was obtained with Cha, N-methylated Phe, Trp, D-Phe, or D-Tyr, full activity was observed only with the L-enantiomer of Phe. Replacement of Phe^6 with Tyr resulted in 75% increase in activity. Only about 10% activity was observed when Ala or His were used.

A basic amino group at the side chain of residue 7 is critical. The length of the side chain did not appear to be important and replacement of Lys with Orn, Dab or Dap resulted in a loss of less than 23% activity. However, substitution with other basic amino acids, Arg or His, resulted in a dramatic loss in activity. Replacement with Ala or an acidic amino acid, Asp, resulted in a total loss of activity.

Table 3 summarizes the results obtained for 10 diastereomeric peptides. It is evident that stereospecificity is crucial at positions 2, 3, and 5, and substitution at these positions with the corresponding D-enantiomer resulted in about 90% loss of activity. In contrast, replacement with a D-amino acid at positions 1, 6, and 7 was less critical, as more than 30% activity still remained. Although Gly is preferred at position 4, substitution with Ala resulted in approximately 66% loss in activity. Replacing Gly^4 with D-Ala, on the other hand, resulted in 98% loss of activity, again indicating the importance of stereospecificity at this position.

DISCUSSION

PTKs have received enormous attention as a major class of enzymes involved in signal transduction and have been strongly implicated in both growth factor and oncogene product action [30]. Unlike many serine/threonine kinases with known substrate motifs [24,25,31], a specific motif has yet to be identified for PTKs. Synthetic peptides based on the primary sequence of the limited number of known protein substrates were ineffi-

TABLE 3
PHOSPHORYLATION SCALE OF YIYGSFK ANALOGUES BY $p60^{c\text{-}src}$ ON SOLID PHASE: SINGLE D-AMINO ACID SUBSTITUTION

Peptide[a]	Phosphorylation scale
YIYGSFK	100
yIYGSFK	65
YiYGSFK	13
YIyGSFK	1
YIYaSFK	2
YIYGsFK	9
YIYGaFK	10
YIYGtFK	12
YIYGSfK	31
YIYGSyK	46
YIYGSFk	56

[a] D-amino acids are denoted by lower-case letters.

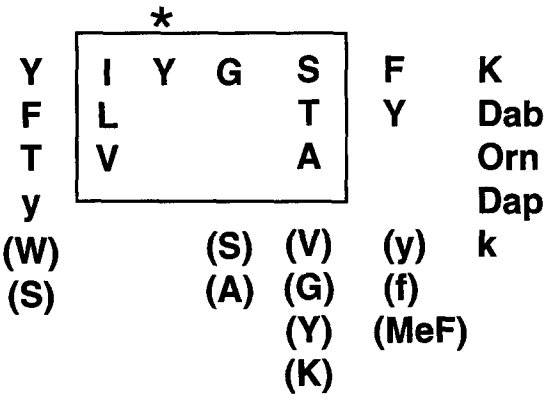

Fig. 2. Summary of the $p60^{c\text{-}src}$ PTK substrate motif: '*' represents site of phosphorylation, residues not in brackets display >55% activity, residues in brackets exhibit 30–55% activity. Stereospecificity is crucial for residues 2–5 (enclosed by the box). See the text for an explanation.

cient substrates for PTK, with K_m in the range 0.5–10 mM [32]. A synthetic peptide cdc2(6–20) derived from $p34^{cdc2}$ was recently shown to be a more specific and efficient substrate for Src-family PTK [33–35]. Very recently, using a combinatorial peptide library method involving affinity selection of a library of solution phosphopeptides, Songyang et al. were able to identify the peptide substrate motifs for several PTKs [27]. These authors incubated a bias random 15-mer peptide library, MAXXXXYXXXXAKKK (where X = all eukaryotic amino acids except Trp, Cys, Tyr, Ser and Thr), with [γ-^{32}P]ATP and $p60^{c\text{-}src}$ PTK. After 2.5 h at 25 °C, the phosphopeptides were isolated by DEAE-Sephacel/ferric chelation column chromatography. The eluted peptides were subjected to concurrent microsequencing. Interestingly, many of the peptides identified were highly acidic and the motif identified for c-Src was EEIYGEFF. Using a different combinatorial library method, in which individual peptide-beads were phosphorylated, isolated, and sequenced, we identified a somewhat different motif, i.e., YIYGSFK. It is important to realize that YIYGSFK can never be isolated from Songyang's bias 15-mer peptide library, as Tyr and Ser were both excluded from their random residues, whereas all 20 eukaryotic amino acids except Cys were included in every coupling cycle of our hepta-peptide library. Although four of the 7–8 amino acids are identical (_ _IYG _ F _), the net charge of the peptides is very different. The EEIYGEFF peptide has three glutamic acid residues and therefore is highly acidic, whereas our peptide, YIYGSFK, is slightly basic. In fact, a basic residue at position 7 is crucial for activity.

In the present study, we synthesized over 70 analogues of YIYGSFK and used the PhosphoImager to quantitate solid-phase phosphorylation of these compounds by $p60^{c\text{-}src}$. This peptide contains two tyrosine residues, Tyr^1 and Tyr^3. The fact that 76%, 71%, 41% or 65% of the activity was retained when Tyr^1 was substituted by Phe, Thr, Trp or D-Tyr makes it unlikely that Tyr^1 is the phosphorylation site. In contrast, when Tyr^3 was replaced by Ala, Phe, or D-Tyr, phosphorylation was almost completely abolished. This strongly suggests that Tyr^3 is the site of phosphorylation. This conclusion was further confirmed by mass spectrometry from our previous study [26]. When Phe^6 was replaced by Tyr, a 75% increase in phosphorylation was found. Whether this is due to an increase in phosphorylation of Tyr^3 or to additional phosphorylation of Tyr^6 is not clear at this time.

Figure 2 summarizes the conclusions obtained from SAR studies of more than 70 analogues of YIYGSFK. It is evident that the core 'IYGS' sequence, as indicated by the box (residues 2–5), is stereospecific and allows for only minor changes if full activity is to be retained. Aromatic residues at positions 1 and 6 are relatively important, but stereospecificity appears not as crucial here. The absolute requirement of a primary amine side chain at the C-terminal residue is intriguing, as the length of the side chain does not appear to be important there (Table 2). Furthermore, 56% activity is still retained when Lys^7 is substituted by its D-enantiomer. However, a guanidinium group (Arg) is not permissable at this position.

Acidic amino acids located at the N-terminal side of the phosphorylation site have been sug-

gested as being important for substrate specificity of several PTKs [1,27,36]. On replacement of Tyr^1 in YIYGSFK by Glu, phosphorylation was reduced to 27%, and when Ile^2 was replaced by Glu, activity was reduced to 6%. Furthermore, when one or more Glu residues were added to the N-terminus of YIYGSFK, phosphorylation also decreased (e.g., to 29% in EYIYGSFK and to 73% in EEEEEEYIYGSFK).

For SAR studies, ideally the peptides should be synthesized and purified, phosphorylation reactions should be carried out in solution phase, and the rate of phosphorylation should be determined. However, it would take enormous effort to synthesize, purify and analyze the large number of peptides described in this paper. As a fast survey, we used the PhosphorImager to quantitate solid-phase phosphorylation of these peptides. The peptides were synthesized on resin beads, where the phosphorylation reaction took place. No purification of peptides is required in this way. Furthermore, separation of the free [γ-^{32}P]ATP from the [^{32}P]-labelled peptides is easy and rapid, as peptides are covalently attached to beads, and phosphorylation can be quantitated by the PhosphorImager. Less than a hundred peptide-beads with a minute amount of enzyme are needed for each assay. Occasional strongly labelled spots in a background of weakly or unlabelled beads were observed on the PhosphorImage, but could easily be identified by dissecting stereomicroscopy as artifacts due to [^{32}P]-impregnated contaminating particles or large bead aggregates (see the two arrows in Fig. 1A). These artifacts would have been read as positive if the labelled beads were counted by liquid scintillation. Using the PhosphorImager, the contaminated particles could be excluded from the quantitative analysis. Quantitation of the solid-phase peptide phosphorylation with the PhosphorImager provides extremely rapid, reproducible, and valuable data as an initial survey for a large number of peptide analogues. Detailed kinetic studies using a solution-phase assay can then be performed on selected peptides.

Combinatorial chemistry, in addition to being a valuable tool for lead discovery, can also be used to optimize the initial leads. Previously, we have successfully used this strategy to optimize the initial lead identified for anti-insulin monoclonal antibody [37]. From this study, we determined that a hydrophobic residue in the –1 position is crucial for $p60^{c\text{-}src}$. We therefore have synthesized and screened a secondary peptide library with the motif XIYXXXX. Preliminary data indicates that more potent and specific substrate can be identified by screening such a secondary library.

CONCLUSIONS

This study demonstrates that SAR studies can be performed rapidly by using a quantitative solid-phase phosphorylation assay. Information obtained from such studies will be useful for understanding the substrate specificity of the important enzyme investigated here. Furthermore, this information may be used in conjunction with computational chemistry for the design of inhibitors for this enzyme. Potent inhibitors for this PTK may have potential therapeutic value, particularly if the substrates are identified from non-peptide (small organic) libraries [38–41], which are more likely to be able to cross the cell membrane than peptides, and can thus interact with the intracellular targets.

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