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Tyr721 regulates specific binding of the CSF-1 receptor kinase insert to PI 3'-kinase SH2 domains: a model for SH2-mediated receptor-target interactions

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Efficient binding of active phosphatidylinositol (PI) 3'-kinase to the autophosphorylated macrophage colony stimulating factor receptor (CSF-1R) requires the non-catalytic kinase insert (KI) region of the receptor. To test whether this region could function independently to bind PI 3'-kinase, the isolated CSF-1R KI was expressed in *Escherichia coli*, and was inducibly phosphorylated on tyrosine. The tyrosine phosphorylated form of the CSF-1R KI bound PI 3'-kinase *in vitro*, whereas the unphosphorylated form had no binding activity. The p85α subunit of PI 3'-kinase contains two Src homology (SH)2 domains, which are implicated in the interactions of signalling proteins with activated receptors. Bacterially expressed p85α SH2 domains complexed *in vitro* with the tyrosine phosphorylated CSF-1R KI. Binding of the CSF-1R KI to PI 3'-kinase activity, and to the p85α SH2 domains, required phosphorylation of Tyr721 within the KI domain, but was independent of phosphorylation at Tyr697 and Tyr706. Tyr721 was also critical for the association of activated CSF-1R with PI 3'-kinase in mammalian cells. Complex formation between the CSF-1R and PI 3'-kinase can therefore be reconstructed *in vitro* in a specific interaction involving the phosphorylated receptor KI and the SH2 domains of p85α.

Key words: colony stimulating factor receptor/kinase insert region/phosphatidylinositol 3'-kinase/phosphorylation/receptor-target binding

Introduction

Activated growth factor receptor tyrosine kinases bind and phosphorylate a set of cytoplasmic signalling proteins, including phospholipase C (PLC)-γ1 and -γ2 (Kumjian *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989; Sultzman *et al.*, 1991), Ras GTPase activating protein (GAP) (Molloy *et al.*, 1989; Ellis *et al.*, 1990; Kazlauskas *et al.*, 1990; Kaplan *et al.*, 1990), phosphatidylinositol (PI) 3'-kinase (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Reedijk *et al.*, 1990; Varticovski *et al.*, 1989) and Src family protein tyrosine kinases (Kypta *et al.*, 1990). These signalling proteins are likely to be the physiological targets which mediate the stimulation of intracellular signal transduction pathways by receptor tyrosine kinases. For example, phosphorylation of PLC-γ1 at Tyr783 is required for induction of its PLC activity in platelet-derived growth factor (PDGF)-stimulated cells (Kim *et al.*, 1991). PLC-γ1, GAP and PI 3'-kinase each possess two copies of a non-catalytic domain, Src homology region (SH)2 (Koch *et al.*, 1991), which appear to direct interactions with activated receptors (Anderson *et al.*, 1990); Src, in contrast, has a single receptor-binding SH2 domain.

Several lines of evidence suggest that receptor autophosphorylation creates high affinity binding sites for SH2-containing proteins. Substitution of the Tyr751 autophosphorylation site in the human βPDGF receptor (PDGFR) with phenylalanine decreases PDGF-induced association with PI 3'-kinase (Kazlauskas and Cooper, 1990; Kazlauskas *et al.*, 1990). A tyrosine phosphorylated peptide spanning the corresponding site of the mouse βPDGFR blocks association of the activated receptor with PI 3'-kinase *in vitro* (Escobedo *et al.*, 1991a). Furthermore, receptor kinase activity is essential for the *in vitro* binding of GAP, PLC-γ1 and PI 3'-kinase SH2 domains to the PDGFR (McGlade *et al.*, 1992). In general, tyrosine phosphorylation of cellular proteins is important for their stable association with SH2 domains *in vitro* (Matsuda *et al.*, 1990; Moran *et al.*, 1990; Mayer *et al.*, 1991).

The autophosphorylation sites of growth factor receptors are frequently contained in non-catalytic regions located within or adjacent to the kinase domain. The PDGFR subfamily of receptor tyrosine kinases, which includes the α and βPDGFRs, the macrophage colony stimulating factor receptor (CSF-1R) and c-Kit, the receptor for Steel factor, each contain a sequence of between 70 and 100 residues which interrupts the kinase domain. This sequence, called the kinase insert region (KI) is dispensable for kinase activity (Taylor *et al.*, 1989; Severinsson *et al.*, 1990; Escobedo and Williams, 1988) but is required for efficient PI 3'-kinase binding (Coughlin *et al.*, 1989; Kazlauskas *et al.*, 1989; Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990; Choudhury *et al.*, 1991). The kinase insert contains demonstrated autophosphorylation sites, including Tyr751 in the human βPDGFR (Kazlauskas *et al.*, 1989), and Tyr697 and Tyr706 in the mouse CSF-1R (Tapley *et al.*, 1990; van der Geer and Hunter, 1990). Tyr751 and surrounding amino acids in the βPDGFR conform to a proposed consensus binding sequence for PI 3'-kinase, consisting of a phosphorylated tyrosine within the context (Asp/Glu)-(Asp/Glu)-P.Tyr-(Met/Val)-(Pro/Asp/Glu)-Met (Cantley *et al.*, 1991). Although they have not been identified as *in vivo* autophosphorylation sites, the sequence surrounding Tyr721 of the mouse CSF-1R KI and Tyr740 of the human βPDGFR KI also conform to this consensus and are presumptive PI

3′-kinase binding sites. Indeed a peptide containing the mouse homologue of human βPDGFR residue 740 inhibits PI 3′-kinase association with the receptor, provided this site is tyrosine phosphorylated (Escobedo *et al.*, 1991a).

PI 3′-kinase (Whitman *et al.*, 1988) is a heterodimer of a regulatory 85 kDa protein (p85), which can bind directly to activated tyrosine kinases (Kaplan *et al.*, 1987; Courtneidge and Heber, 1987) and a 110 kDa polypeptide which may be the catalytic subunit (Carpenter *et al.*, 1990; Morgan *et al.*, 1990). Stimulation of cells with PDGF or CSF-1 leads to the formation of phosphoinositides which are phosphorylated at the D-3 position of the inositol ring, suggesting that these growth factors stimulate PI 3′-kinase activity *in vivo* (Auger *et al.*, 1989; Varticovski *et al.*, 1989).

cDNAs for two isoforms of p85 have been sequenced, and shown to encode closely related proteins with two SH2 domains (Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Skolnik *et al.*, 1991). Since deletion of the CSF-1R KI results in a profound decrease in the ability of the receptor to associate with PI 3′-kinase in CSF-1 stimulated cells (Reedijk *et al.*, 1990; Shurtleff *et al.*, 1990; Choudhury *et al.*, 1991), we have tested the possibility that the CSF-1R KI is sufficient for PI 3′-kinase binding, and have investigated the roles of receptor phosphorylation and the p85α SH2 domains in these interactions.

Results

Phosphorylation-dependent binding of the isolated CSF-1R KI to PI 3′-kinase

To investigate the binding properties of the CSF-1R KI, we expressed this domain of the receptor in bacteria, and assessed its ability to bind PI 3′-kinase. For this purpose a sequence encoding 139 residues of the mouse CSF-1R, which includes the entire KI, was excised from the c-*fms* cDNA (Rothwell and Rohrschreider, 1987) and expressed as a TrpE fusion protein in *Escherichia coli* (TrpE−KI). To induce tyrosine phosphorylation of the TrpE−KI fusion protein, bacteria harboring the TrpE−KI plasmid were infected with a λgt11 bacteriophage (λB1-Elk) encoding the cytoplasmic domain of the Elk tyrosine kinase, which is extremely active in *E.coli* (Letwin *et al.*, 1988; Lhotak *et al.*, 1991). Bacteria containing the TrpE−KI plasmid and the λB1-Elk bacteriophage as a lysogen were isolated. Following induction of the λB1-Elk lysogen, the TrpE−KI fusion protein became phosphorylated (Figure 1A, lane 3). Phosphoamino acid analysis of the TrpE−KI protein immunoprecipitated from ^{32}P-labelled *E.coli* expressing the Elk tyrosine kinase revealed phosphotyrosine as the only detectable phosphoamino acid (Figure 1B).

To test for PI 3′-kinase binding activity, the unphosphorylated or tyrosine phosphorylated TrpE−KI fusion proteins were isolated by immunoprecipitation of unlabelled bacterial extracts, incubated with lysates of Rat-2 fibroblasts, washed and assayed for associated PI 3′-kinase activity (Figure 2). The unphosphorylated TrpE−KI protein showed no binding activity. In contrast the tyrosine phosphorylated TrpE−KI bound PI 3′-kinase activity when incubated with a Rat-2 cell lysate.

To investigate whether PI 3′-kinase binding is a specific property of the KI, a 76 amino acid C-terminal fragment of the CSF-1R was also expressed in *E.coli* as a TrpE fusion

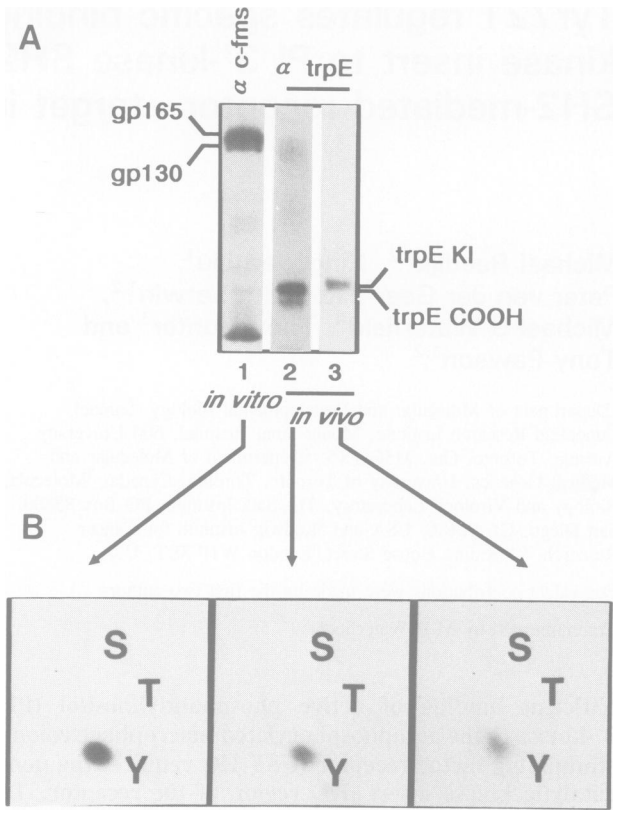

Fig. 1. Expression and tyrosine phosphorylation of the CSF-1R KI in *E.coli*. (**A**) *E.coli* expressing TrpE fused either to a region containing the mouse CSF-1R KI (TrpE−KI) or to the CSF-1R C-terminal region (TrpE−COOH) were superinfected with the λB1-Elk bacteriophage, encoding the Elk tyrosine kinase domain, and lysogens were isolated. *E.coli* were labelled with [^{32}P]orthophosphate, and the TrpE fusion proteins and the Elk tyrosine kinsae were sequentially induced. Labelled bacterial lysates were immunoprecipitated with anti-TrpE antibodies (αTrpE), and the immune complexes were separated by polyacrylamide gel electrophoresis, transferred to an Immobilon membrane, and subjected to autoradiography (TrpE−COOH, lane 2; TrpE−KI, lane 3). WT mouse CSF-1R (gp165, gp130) was immunoprecipitated from Rat-2 cells expressing c-*fms* with anti-Fms antibodies, and autophosphorylated *in vitro* using [γ-^{32}P]ATP (lane 1). (**B**) ^{32}P-Labelled CSF-1R, TrpE−KI and TrpE−COOH were excised from the membrane and were analyzed for phosphoamino acid content. The positions of unlabelled phosphoamino acid markers are shown: phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y).

protein (TrpE−COOH). When the TrpE−COOH protein was co-expressed with the λB1-Elk tyrosine kinase it became extensively tyrosine phosphorylated, albeit at sites which may be non-physiological (Figure 1). However, neither the unphosphorylated nor the tyrosine phosphorylated TrpE−COOH proteins were able to bind PI 3′-kinase in a Rat-2 cell lysate (Figure 2). Hence PI 3′-kinase binding requires tyrosine phosphorylation within a specific context provided by the KI.

Binding of PI 3′-kinase to bacterially expressed KI requires phosphorylation of CSF-1R Tyr721

The identity of the tyrosine residue(s) necessary for association of the CSF-1R KI with PI 3′-kinase was addressed by substituting each of the three tyrosine residues in the KI, Tyr697, Tyr706 and Tyr721, with phenylalanine (Phe) in the TrpE−KI fusion protein. The mutant proteins were co-expressed in *E.coli* with the λB1-Elk tyrosine kinase,

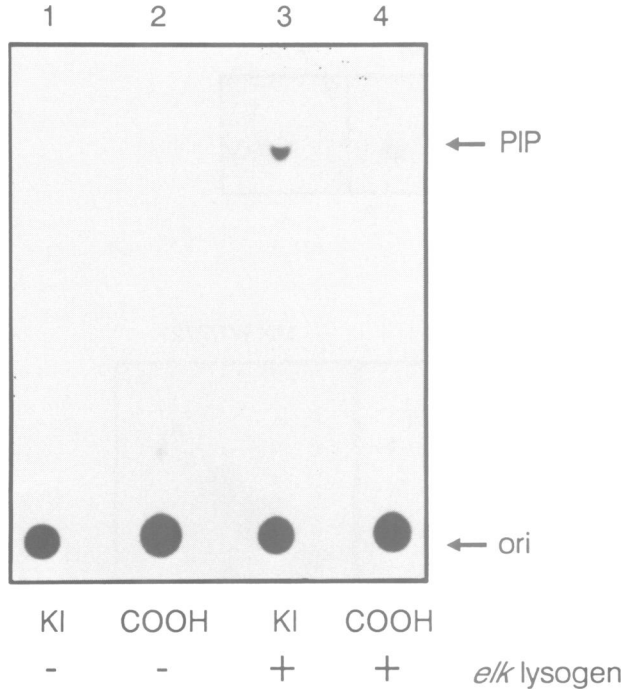

Fig. 2. Association of the tyrosine phosphorylated CSF-1R KI with PI 3′-kinase. Bacterial TrpE fusion proteins were isolated and tested for their ability to bind PI 3′-kinase in a lysate of Rat-2 fibroblasts. Either non-phosphorylated (−*elk* lysogen, lanes 1 and 2) or tyrosine phosphorylated (+*elk* lysogen, lanes 3 and 4) TrpE fusion proteins (TrpE−KI, lanes 1 and 3; TrpE−COOH, lanes 2 and 4) were isolated from bacterial lysates with anti-TrpE antibodies, and immobilized on protein A−Sepharose beads. 2×10^6 Rat-2 cells were lysed as described previously (Reedijk *et al.*, 1990), and incubated with the immobilized TrpE fusion proteins for 1 h at 4°C. Complexes were then washed, and tested for associated PI 3′-kinase activity. ori, origin. PIP, phosphatidylinositol monophosphate.

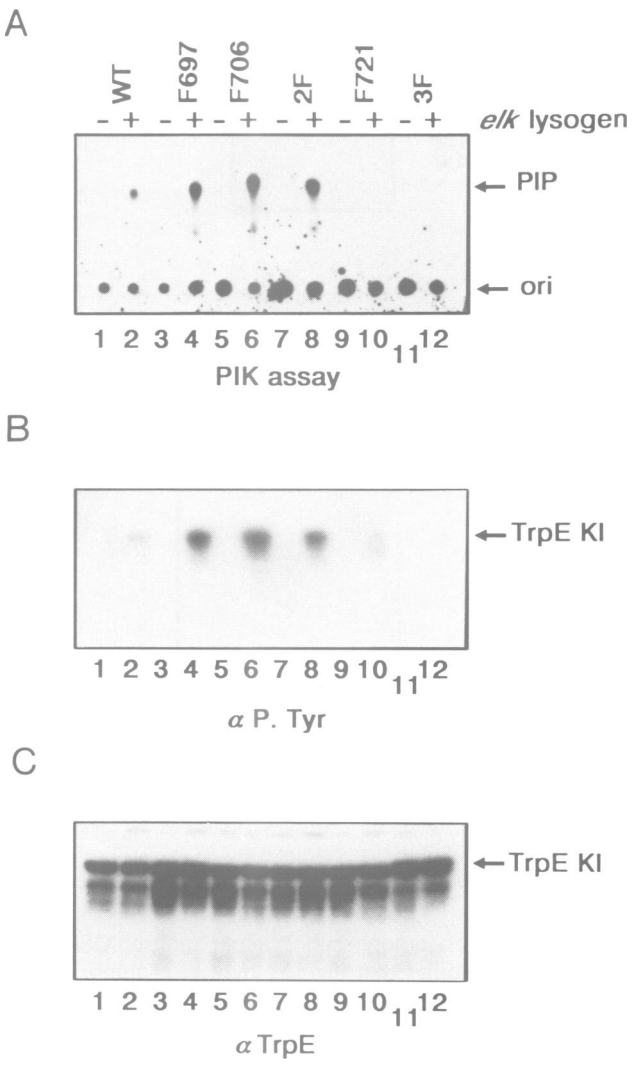

Fig. 3. Substitution of CSF-1R Tyr721 with phenylalanine abolishes *in vitro* association of the KI with PI 3′-kinase. TrpE−KI fusion proteins containing tyrosine to phenylalanine mutations at various locations within the KI were expressed in *E.coli* and were either left in their non-phosphorylated state (lanes 1, 3, 5, 7, 9 and 11) or were phosphorylated on tyrosine (lanes 2, 4, 6, 8, 10 and 12). (**A**) Fusion proteins, immobilized with anti-TrpE antibodies and protein A−agarose, were incubated with Rat-2 cell lysates. The complexes were then analyzed for associated PI 3′-kinase activity. WT, wild-type insert (lanes 1 and 2); F697, Phe697 mutant (lanes 3 and 4); F706, Phe706 mutant (lanes 5 and 6); 2F, Phe697/Phe706 double mutant (lanes 7 and 8); F721, Phe721 mutant (lanes 9 and 10); 3F, Phe697/Phe706/Phe721 triple mutant (lanes 11 and 12). Anti-TrpE immunoprecipitates were also subjected to Western blotting analysis with either anti-phosphotyrosine antibodies (α P. Tyr) (**B**), or anti-TrpE antibodies (**C**) followed by [^{125}I]protein A.

and analyzed for their tyrosine phosphorylation and PI 3′-kinase binding activity (Figure 3). Equivalent amounts of TrpE fusion proteins were isolated in each case (Figure 3C). TrpE−KI proteins with Phe at positions 697 (F697) or 706 (F706), or with Phe at both 697 and 706 (2F), were more highly tyrosine phosphorylated than the wild-type (WT) fusion protein (Figure 3B), and bound concomitantly more PI 3′-kinase when incubated with a Rat-2 cell lysate (Figure 3A). However, the Phe721 mutant TrpE−KI (F721), although it was still tyrosine phosphorylated, was defective in PI 3′-kinase association. A TrpE−KI protein with Phe at positions 697, 706 and 721 (3F) did not become detectably tyrosine phosphorylated and failed to bind PI 3′-kinase. These results suggest that phosphorylation of Tyr721 allows the bacterial fusion protein to bind PI 3′-kinase.

To investigate which tyrosine residues were phosphorylated in the WT and mutant bacterial proteins, TrpE−KI proteins were immunoprecipitated from lysates of ^{32}P-labelled bacteria, following induction of the Elk tyrosine kinase, and subjected to both phosphoamino acid analysis and two-dimensional tryptic phosphopeptide analysis. Both the WT and F721 proteins were phosphorylated exclusively on tyrosine, whereas the 3F mutant did not become ^{32}P-labelled (Figure 4A and B). The WT TrpE−KI gave three major tryptic phosphopeptides (labelled 1, 2 and 3 in Figure 4C), and several minor ones, including those identified as A, B and C. The major spots 1−3 were lost in the F721 TrpE−KI (Figure 4C), but were present in maps of the other mutant proteins (F697, F706, 2F; data not shown), suggesting that these spots represent peptides containing phosphorylated Tyr721. Tyr721 is flanked on its N-terminal side by two adjacent arginine residues (Arg708, Arg709), and on its C-terminal side by an arginine (Arg725) followed by proline; differential tryptic cleavage at these basic residues could account for the multiple spots apparently containing phosphorylated Tyr721. Peptide 1 of WT TrpE−KI co-migrated with a minor spot (peptide d) in a

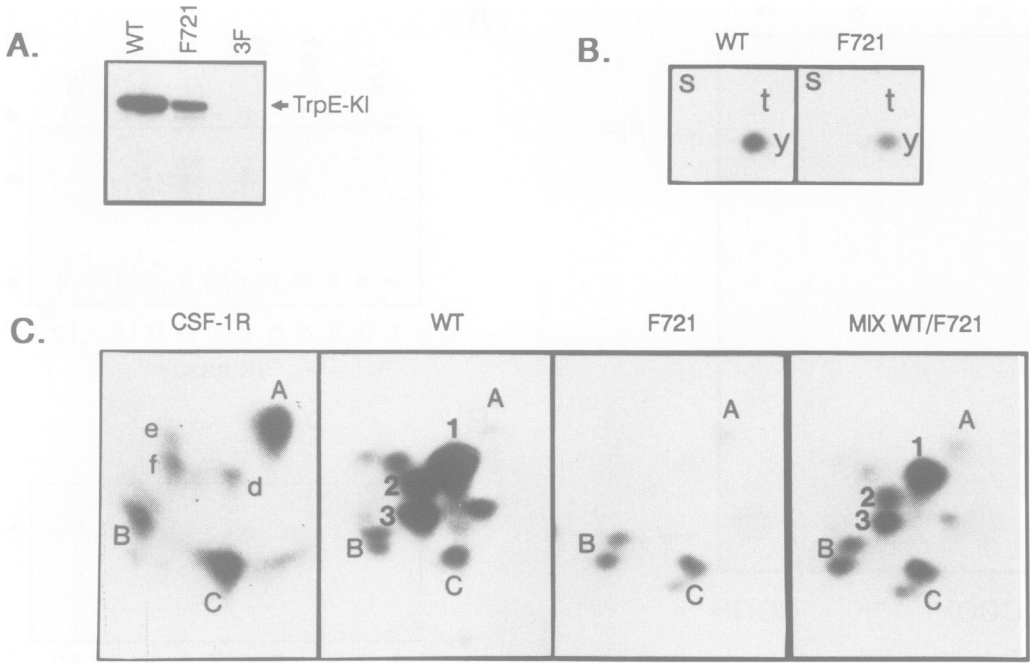

Fig. 4. Tyr721 in the TrpE−KI fusion protein is phosphorylated in bacteria. (**A**) Bacterial cultures containing WT, F721, or 3F TrpE−KI proteins and the λB1-Elk tyrosine kinase were labelled with ^{32}P; lysed, and immunoprecipitated with 2 μg of monoclonal anti-TrpE antibodies bound to goat anti-mouse IgG agarose. The bound proteins were separated on a 12% SDS−polyacrylamide gel and analyzed by autoradiography. (**B**) ^{32}P-Labelled WT and F721 TrpE−KI fusion proteins were subjected to phosphoamino acid analysis. The positions of unlabelled phosphoserine (s), phosphothreonine (t) and phosphotyrosine (y) markers are indicated. (**C**) ^{32}P-Labelled WT and F721 TrpE−KI fusion proteins, phosphorylated by the Elk tyrosine kinase in *E.coli* were digested with trypsin. In addition, the full-length CSF-1R was autophosphorylated *in vitro* with [γ-^{32}P]ATP and subjected to trypsin digestion. The resulting tryptic phosphopeptides were separated by electrophoresis at pH 2.1 followed by ascending chromatography, and identified by autoradiography. The indicated spots are discussed in the text.

tryptic digest of autophosphorylated mouse CSF-1R, raising the possibility that Tyr721 may be a minor autophosphorylation site of the activated full-length receptor *in vitro*. Phosphopeptides A, B and C were minor spots in WT (Figure 4C), but became more apparent in F721. Peptide A was absent from a digest of the F706 protein (data not shown), suggesting that this peptide contains phosphorylated Tyr706. Spots B and C were missing in the map of the F697 protein (data not shown), indicating that these spots are derived from a Tyr697-containing phosphopeptide. Furthermore, peptides A, B and C from the F721 protein co-migrated with phosphopeptides from autophosphorylated mouse CSF-1R (Figure 4C and data not shown) that have been previously shown to contain Tyr706 (spot A) and Tyr697 (spots B and C) (Tapley *et al.*, 1990; van der Geer and Hunter, 1990). These results confirm that phosphorylation of Tyr721 in bacteria endows TrpE−KI with the ability to bind PI 3′-kinase, whereas phosphorylation of Tyr697 and Tyr706 does not contribute to PI 3′-kinase binding.

Binding of p85 SH2 domains to the CSF-1R KI is regulated by phosphorylation of Tyr721

Since the p85 SH2 domains are strong candidates for the elements that direct PI 3′-kinase association with activated receptors, we tested the ability of p85α SH2 domains to bind the TrpE−KI polypeptide (Figure 5). The N-terminal (N) and C-terminal (C) p85α SH2 domains were independently expressed in *E.coli* as glutathione *S*-transferase (GST) fusion proteins, and immobilized by attachment to glutathione−agarose beads. The immobilized GST−SH2 fusion proteins were then incubated with lysates of bacteria expressing the TrpE−KI in its phosphorylated or unphosphorylated form. The complexes were washed, and then analyzed by immunoblotting with anti-TrpE antibodies to detect any TrpE−KI that had associated with the PI 3′-kinase GST−SH2 fusion proteins. Very little of the unphosphorylated TrpE−KI protein complexed with the immobilized p85α SH2 fusion proteins. However, tyrosine phosphorylation of the TrpE−KI induced marked binding to the p85α SH2 domains. The C-terminal p85α SH2 domain consistently complexed more efficiently with the phosphorylated TrpE−KI than did the N-terminal SH2 domain. A fusion protein containing both the N and C SH2 domains (N + C) bound the tyrosine phosphorylated KI with greater affinity than did either the N or C SH2 domains alone (Figure 6). Synergistic binding of SH2 domains to receptors has been noted previously for PLC-γ1 and GAP (Anderson *et al.*, 1990).

In contrast to the SH2 domains of PI 3′-kinase, no complex formation was observed between the N-terminal GAP SH2 domain and the CSF-1R KI, even when the latter was tyrosine phosphorylated. This is consistent with the observation that GAP does not bind efficiently to the CSF-1R *in vivo* (Reedijk *et al.*, 1990; M.Reedijk, unpublished results).

The importance of CSF-1R Tyr721 in the binding of p85α SH2 domains was investigated by incubating GST fusion proteins containing the N- or C-terminal SH2 domains, or both SH2 domains together, with lysates of bacteria expressing either the WT, 2F or F721 TrpE−KI proteins in their tyrosine phosphorylated forms (Figure 6). The 2F

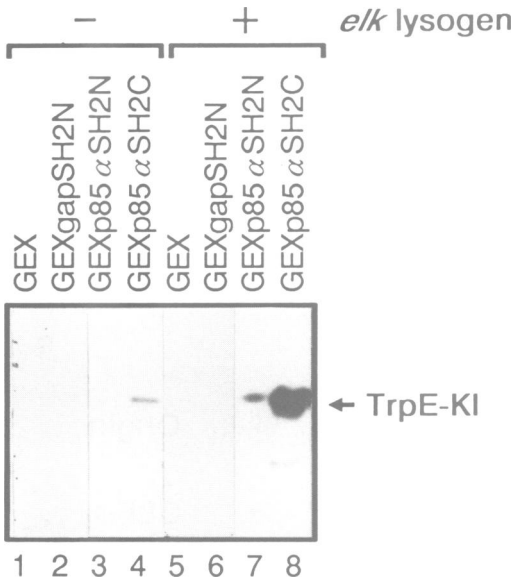

Fig. 5. The SH2 domains of the p85α subunit of PI 3′-kinase bind specifically to the tyrosine phosphorylated CSF-1R KI. Bacteria expressing GST alone (GEX; lanes 1 and 5), or GST fusion proteins containing the N-terminal GAP SH2 domain (GEXgapSH2N; lanes 2 and 6), the N-terminal p85α SH2 domain (GEXp85αSH2N; lanes 3 and 7), and the C-terminal p85α SH2 domain (GEXp85αSH2C; lanes 4 and 8) were lysed, and the GST fusion proteins were collected on glutathione–agarose beads. The beads were resuspended in lysates from bacteria expressing WT TrpE–KI either in its phosphorylated (lanes 5–8) or non-phosphorylated (lanes 1–4) form. After 1.5 h incubation at 4°C, the beads were washed and associated proteins were electrophoresed through a 12.5% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with αTrpE antibodies, followed by [^{125}I]protein A to detect SH2-associated TrpE–KI fusion proteins.

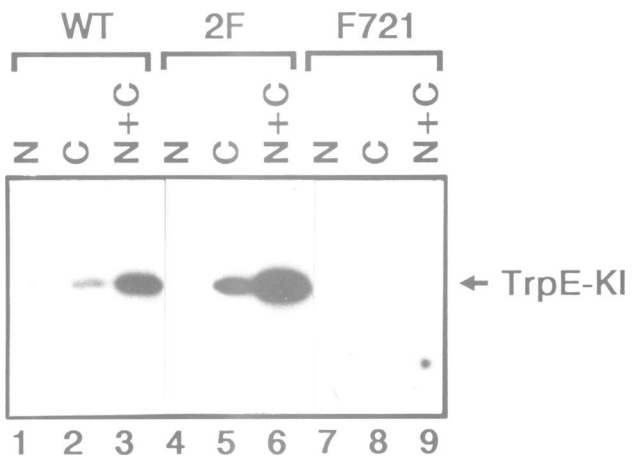

Fig. 6. Binding of p85α SH2 domains to the CSF-1R KI requires phosphorylation of Tyr721. Fusion proteins encoded by GEXp85αSH2N (lanes 1, 4 and 7), GEXp85αSH2C (lanes 2, 5 and 8) and GEXp85αSH2N+C (GST fused to both p85α SH2 domains; lanes 3, 6 and 9) were immobilized on glutathione–agarose beads. The beads were resuspended in lysates of bacteria expressing λB1-Elk in combination with either WT TrpE–KI (lanes 1–3), 2F (lanes 4–6) or F721 (lanes 7–9). The association of the WT or mutant forms of the KI with p85α SH2 domains was analyzed by immunoblotting with anti-TrpE antibodies, followed by [^{125}I]protein A.

TrpE–KI mutant, in which Tyr697 and Tyr706 are replaced by Phe, bound the p85α SH2 domains with an efficiency similar to that of the WT KI. In contrast, the F721 TrpE–KI mutant did not bind a detectable amount of p85α SH2 domains. This result is in agreement with the observation that F721 was severely reduced in its ability to complex with PI 3′-kinase activity *in vitro* (Figure 3) and *in vivo* (see below).

Binding of PI 3′-kinase to the CSF-1R in CSF-1-stimulated cells is dependent on Tyr721

The possible role of different tyrosine autophosphorylation sites in the association of the CSF-1R with PI 3′-kinase *in vivo* was studied by expressing WT and mutant mouse CSF-1R cDNAs in 208F rat fibroblasts. Mutations which convert the codons for CSF-1R Tyr697, Tyr706 and Tyr721, either alone or in combination, were introduced into a full-length CSF-1R cDNA. A retroviral vector was then employed to obtain stable cell lines expressing the WT or mutant receptors. These cells were serum-starved for 48 h, and then stimulated with recombinant human CSF-1 (rhCSF-1R). CSF-1R immunoprecipitates from control and CSF-1-stimulated cells were tested for associated PI 3′-kinase activity (Figure 7A). To control for CSF-1R levels, receptor immunoprecipitates from parallel dishes were analyzed by immunoblotting with an anti-CSF-1R serum (Figure 7B). Whereas WT, F697, F706 and 2F (F697, F706) mutant receptors all associated with PI 3′-kinase upon stimulation with CSF-1, the F721 and 3F (F697, F706, F721) mutant receptors failed to do so. These results confirm our *in vitro* association data, and suggest that Tyr721 in the murine CSF-1R is essential for CSF-1-dependent association of the receptor with PI 3′-kinase *in vivo*.

Discussion

Phosphorylation of Tyr721 within the CSF-1R KI forms a discrete binding site for PI 3′-kinase

Autophosphorylation of growth factor receptors has been implicated in the binding of SH2-containing signalling proteins (Margolis *et al.*, 1990). In particular, tyrosine phosphorylation within the KI of the PDGFR subfamily of receptors has been identified as providing likely binding sites for signalling proteins such as PI 3′-kinase. These observations suggest that the KI forms a discrete non-catalytic domain that serves to recruit signalling proteins to the activated receptors. To test this notion we have expressed the KI on its own in bacteria and shown that it possesses an intrinsic ability to bind PI 3′-kinase, which is greatly enhanced when it is phosphorylated on tyrosine. Mutagenesis studies indicate that phosphorylation of Tyr721, within a predicted binding consensus sequence, is critical for PI 3′-kinase association. Consistent with this observation, preincubation of activated CSF-1R with antibodies raised against a KI peptide containing residues corresponding to 699–719 in mouse CSF-1R reduces the ability of the receptor to associate with PI 3′-kinase (Downing *et al.*, 1991).

Phosphopeptide mapping of the WT and mutant TrpE–KI proteins digested with trypsin has suggested that Tyr721 does become phosphorylated by the λB1-Elk tyrosine kinase in *E.coli*. Although Tyr721 has not been observed to be phosphorylated in mammalian cells in response to CSF-1, it is quite possible that the phosphorylated site is very

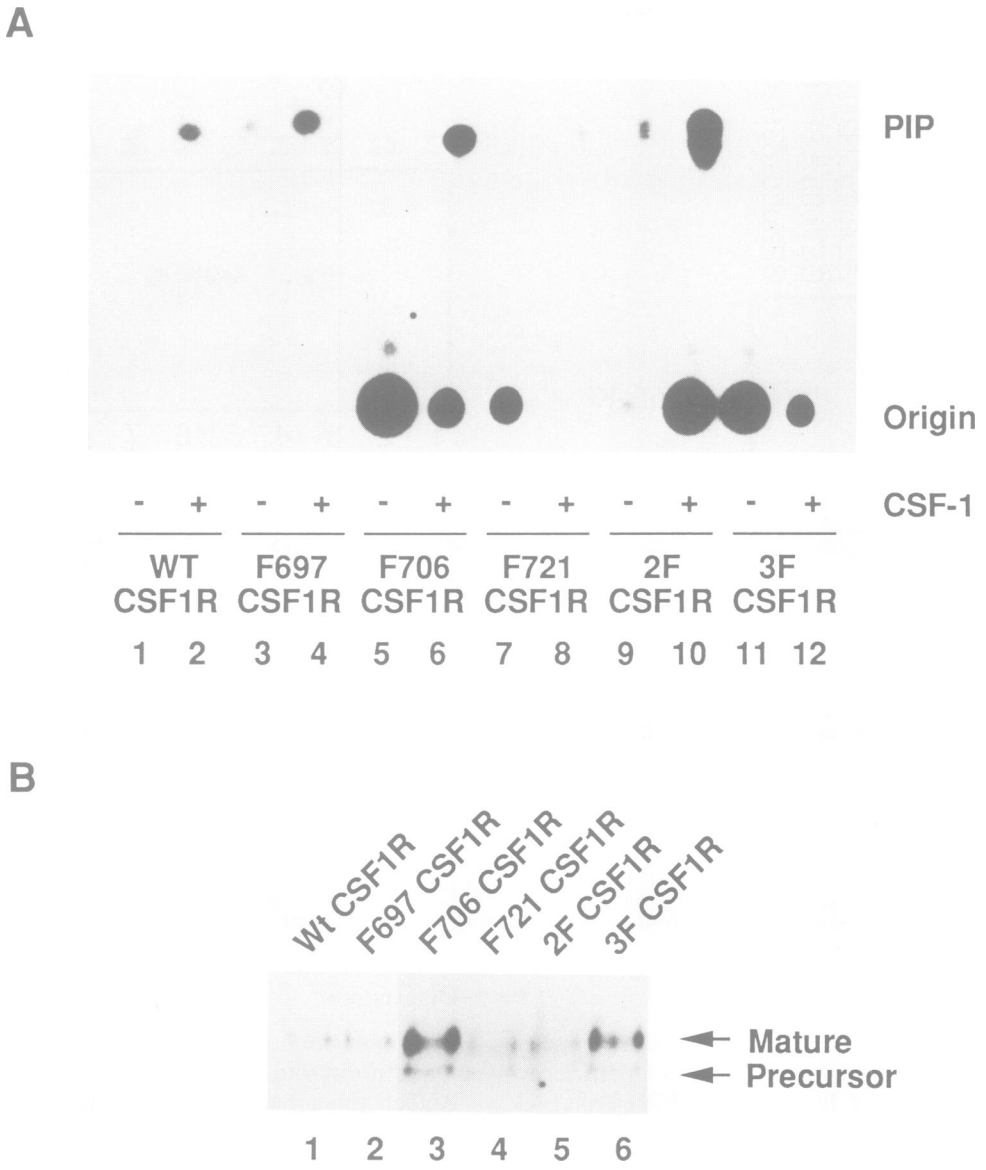

Fig. 7. Substitution of Tyr721 in the murine CSF-1R with phenylalanine abolishes CSF-1 induced association of the receptor with PI 3′-kinase activity *in vivo*. Wild-type (WT) or mutant CSF-1R cDNAs were stably expressed in rat fibroblasts as described in Materials and methods. (**A**) Confluent 10 cm tissue culture dishes of 208F cells expressing WT and mutant CSF-1R cDNAs, as indicated, were starved in DMEM plus 10 mM HEPES, pH 7.4, and stimulated with 100 ng/ml rhCSF-1 for 3 min at 37°C. CSF-1R immunoprecipitates from control cells (lanes 1, 3, 5, 7, 9 and 11) and CSF-1 stimulated cells (lanes 2, 4, 6, 8, 10 and 12) were tested for associated PI 3′-kinase activity (van der Geer and Hunter, 1990). (**B**) Receptor immunoprecipitates obtained from parallel dishes were separated by SDS−PAGE, transferred to Immobilon P and immunoblotted with anti-CSF-1R antibodies to quantify CSF-1R levels as described (van der Geer and Hunter, 1991). PIP, phosphatidylinositol monophosphate.

sensitive to phosphotyrosine phosphatases, and is rapidly dephosphorylated unless it becomes occupied by an SH2 domain. Since the fraction of activated CSF-1R that associates with PI 3′-kinase *in vivo* is low (Downing *et al.*, 1991), the extent of Tyr721 phosphorylation *in vivo* might be correspondingly modest and below the normal limits of detection. These conclusions receive support from the observation that a Phe721 mutant CSF-1R fails to bind PI 3′-kinase in 208F cells following CSF-1 stimulation, whereas the Phe697 and Phe706 mutants show WT levels of binding.

p85 SH2 domains confer specific binding to the Tyr721 site of the CSF-1R

Since the p85 receptor binding subunit of PI 3′-kinase contains two SH2 domains, it seemed likely that these would mediate the interaction of PI 3′-kinase with the CSF-1R KI. Indeed, we found that the tyrosine phosphorylated CSF-1R KI bound directly to the SH2 domains of the p85α regulatory subunit of PI 3′-kinase. In these experiments, both the CSF-1R KI and the p85α SH2 domains were expressed and isolated as bacterial fusion proteins. Hence, the phosphorylated KI of the CSF-1R and the SH2 domains of p85 contain all the structural information required for the formation of a stable complex. The CSF-1R and PI 3′-kinase have therefore evolved complementary non-catalytic domains that allow for their mutual, phosphorylation-dependent association. This is likely to be a general feature of the interactions of receptor tyrosine kinases with their targets.

Efficient *in vitro* binding of p85α SH2 domains is induced by tyrosine phosphorylation of the KI. In particular, phosphorylation of Tyr721 within the KI is critical for SH2 binding, whereas phosphorylation of Tyr697 and Tyr706 is

neither necessary nor sufficient for stable association with the p85α SH2 domains. These results imply that the ability of PI 3′-kinase to bind to the phosphorylated CSF-1R *in vivo* depends on the recognition of a site encompassing Tyr721 by the p85 SH2 domains. The specificity with which PI 3′-kinase binds to this site is apparently an intrinsic property of the p85α SH2 domains. A phylogenetic tree of known SH2 sequences (data not shown) indicates that the two SH2 domains of p85 are more closely related to one another than to other SH2 domains, consistent with the observation that they possess related binding specificities.

How might an SH2 domain discriminate between different tyrosine phosphorylated sites? In addition to a predicted interaction between conserved, SH2 basic residues and phosphotyrosine (Koch *et al.*, 1991), there are likely to be specific contacts between more variable SH2 residues and the amino acids surrounding receptor tyrosine phosphorylation sites. Such interactions may allow PI 3′-kinase to bind more strongly to the phosphorylated sites encompassing Tyr721, than to the sites containing Tyr697 or Tyr706. Whether these latter sites are recognized by the SH2 domains of other, as yet unidentified signalling proteins remains to be seen. It will be of considerable interest to identify those residues in SH2 domains which confer specificity for one binding site over another. This may ultimately allow the interactions of receptors and SH2-containing signalling proteins to be inferred solely from their sequences.

Materials and methods

Construction of bacterial expression vectors

A 415 bp *Hin*cII restriction fragment of murine c-*fms* cDNA encoding residues 646–784 was ligated in-frame into the bacterial expression plasmid pATH-2. Expression of this plasmid in *E.coli* yields a protein (WT TrpE–KI) in which TrpE is fused with a 139 amino acid fragment of the CSF-1R cytoplasmic domain containing the KI. Similar plasmids were constructed in which the *Hin*cII restriction fragments encoded a variety of Tyr → Phe mutations within the KI (F697, Phe697 mutant; F706, Phe706 mutant; F721, Phe721 mutant; 2F, Phe697 and Phe706 double mutant; 3F, Phe697, Phe706 and Phe721 triple mutant). TrpE–COOH encodes a TrpE protein fused in-frame to amino acids 901–976 of the CSF-1R.

Sequences encoding the N-terminal (residues 312–444) or C-terminal (residues 612–722) SH2 domains of bovine p85α (Otsu *et al.*, 1991) were amplified using the polymerase chain reaction (PCR) and cloned in-frame with the glutathione *S*-transferase (GST) sequences of plasmid pGEX2t to produce the consructs GEXp85αSH2N or GEXp85αSH2C respectively. A larger PCR-amplified fragment encoding both SH2 domains (residues 312–722) was also ligated into the vector to generate GEXp85SH2N + C. Similarly, human GAP N-terminal SH2 sequences (amino acids 178–278 of human GAP) were cloned into pGEX2t to produce GEXgapSH2N.

Generation of bacterial clones co-expressing TrpE fusion proteins and the Elk tyrosine kinase

Escherichia coli containing various pATH expression plasmids were infected with a λ phage (λB1) encoding the cytoplasmic domain of the Elk tyrosine kinase as a LacZ–Elk fusion (Letwin *et al.*, 1988). Infected bacteria were plated at 30°C. Single colonies were isolated and plated in duplicate at 30° and 42°C. Clones carrying the λB1 lysogen only grew at 30°C and lysed at 42°C. The TrpE fusion proteins and the Elk tyrosine kinase were sequentially induced to achieve Elk-dependent tyrosine phosphorylation of TrpE fusion proteins. Following induction of TrpE fusion proteins at 30°C (Moran *et al.*, 1990), the bacterial cultures were heat shocked at 42°C followed by a 1 h incubation with 1 mM isopropyl-β-D-thiogalactoside (IPTG) at 37°C to induce the Elk kinase, and the consequent phosphorylation of the TrpE fusion proteins.

In vitro binding of TrpE–KI fusion proteins to PI 3′-kinase and to GST–SH2 fusions

Bacteria containing phosphorylated TrpE–KI from a 10 ml culture were pelleted and resuspended in 500 μl of PLC lysis buffer [50 mM *N*-2 hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM pyrophosphate and 100 mM sodium fluoride]. The suspensions were sonicated and clarified by centrifugation. Clarified lysates were incubated for 90 min at 4°C with rabbit anti-TrpE antibodies (1 μg/ml) and 100 μl protein A–Sepharose. Immunoprecipitates were washed three times with the lysis buffer and resuspended in a lysate from 2 × 10^6 Rat-2 cells and allowed to incubate for 60 min at 4°C. Rat-2 cells were lysed as described in Reedijk *et al.* (1990). Immunoprecipitates were re-washed and analyzed for associated PI kinase activity as described (Fukui and Hanafusa, 1989). Aliquots of the immunoprecipitates were analyzed by immunoblotting with anti-TrpE or anti-phosphotyrosine antibodies (Moran *et al.*, 1990).

GST fusion proteins containing p85α or GAP SH2 domains were induced with 1 mM IPTG. Bacterial lysates were prepared as above and the fusion proteins were immobilized with glutathione–agarose beads. These immobilized SH2 domains were incubated with bacterial lysates containing TrpE–KI (or its various Tyr → Phe mutants) either in their phosphorylated or non-phosphorylated form. After 1.5 h incubation at 4°C, the beads were washed and associated proteins were separated on a 12.5% polyacrylamide gel and transferred to nitrocellulose. Bound TrpE–KI fusion proteins were detected with anti-TrpE antibodies followed by [^{125}I]protein A.

Phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping

Following induction of TrpE–KI or TrpE–COOH fusion proteins at 30°C, bacterial cells from a 5 ml culture were pelleted and washed twice with phosphate-free Dulbecco's modified Eagle's medium. The washed cells were resuspended in 1 ml of the same medium and heat shocked at 42°C for 15 min. 1 mCi of [^{32}P]orthophosphate was added to the medium followed by IPTG to a final concentration of 1 mM. After 1 h incubation at 37°C the cells were pelleted and washed with 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 0.3 M NaCl. The washed cells were lysed by incubation in 100 μl SDS-cracking buffer (10 mM Na phosphate, pH 7.5, 1% β-mercaptoethanol, 1% SDS, 6 M urea) for 3 h at 37°C. The lysate was diluted 20-fold with PLC buffer and clarified by centrifugation. ^{32}P-Labelled TrpE–KI fusion proteins were isolated with anti-TrpE antibodies, separated by SDS–PAGE, and transferred to an Immobilon membrane. Immobilon strips containing the fusion proteins were excised, and the phosphoamino acid content of the ^{32}P-labelled proteins was analyzed by acid hydrolysis, followed by two-dimensional electrophoresis at pH 1.9 and pH 3.5 (Cooper *et al.*, 1983). For peptide mapping, ^{32}P-labelled WT, F721, or 3F TrpE–KI proteins were immunoprecipitated with mouse monoclonal anti-TrpE antibodies bound to goat anti-mouse IgG agarose, and digested with trypsin. The full-length CSF-1R was immunoprecipitated from Rat-2 fibroblast cell line over-expressing c-*fms* with anti-Fms antiserum (Reedijk *et al.*, 1990), and autophosphorylated *in vitro* in the presence of [γ-^{32}P]ATP. Trypsin digestion of the full-length CSF-1R was performed on the nitrocellulose strip (Liu and Pawson, 1991). All resulting peptides were separated by electrophoresis at pH 2.1, followed by ascending chromatography (Liu and Pawson, 1991).

In vivo association of WT and mutant CSF-1R with PI 3′-kinase

WT and mutant CSF-1R cDNAs were cloned into an MuLV-based retroviral expression vector (Scharfmann *et al.*, 1991). PA12 packaging cells were transfected as described (van der Geer and Hunter, 1991). Supernatants were taken 48 h after transfecton and were used to infect 208F rat fibroblasts. Cells were selected for expression of the neomycin resistance gene in DMEM plus 400 μg/ml G418. Confluent 10 cm tissue culture dishes of 208F cells expressing WT and mutant CSF-1R cDNAs were starved in DMEM plus 10 mM HEPES, pH 7.4, and stimulated with 100 ng/ml rhCSF-1 for 3 min at 37°C. CSF-1R immunoprecipitates from control and CSF-1 stimulated cells were tested for PI 3′-kinase activity. Receptor immunoprecipitates obtained from parallel dishes were separated by SDS–PAGE, transferred to Immobilon P and probed for CSF-1R level as described before (van der Geer and Hunter, 1991).

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