Functional Interaction Trap
A STRATEGY FOR VALIDATING THE FUNCTIONAL CONSEQUENCES OF TYROSINE PHOSPHORYLATION OF SPECIFIC SUBSTRATES IN VIVO

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Protein tyrosine phosphorylation controls diverse signaling pathways, and deregulated tyrosine kinase activity plays a direct role in human diseases such as cancer. Because activated kinases exert their effects by phosphorylating multiple substrate proteins, it is difficult or impossible to assess experimentally the contribution of a particular substrate to a cellular response or activity. To overcome this problem, we have developed a novel approach termed the "functional interaction trap," in which two proteins are induced to interact in a pairwise fashion through an engineered, highly specific binding interface. We show that the functional interaction trap can be used to direct a modified tyrosine kinase to specifically phosphorylate a single substrate of choice in vivo, permitting analysis of the resulting biological output. This strategy provides a powerful tool for validating the functional significance of tyrosine phosphorylation and other post-translational modifications identified by proteomic discovery efforts. Molecular & Cellular Proteomics 2: 1217–1224, 2003.

Protein-tyrosine kinases play a central role in cellular signal transduction, regulating many activities of direct relevance to human disease (1). Accordingly, there is a considerable interest in proteomic efforts to profile and identify tyrosine-phosphorylated proteins under various physiological conditions (2–5). Non-receptor tyrosine kinases such as Src bind via their Src homology (SH)1 and SH2 protein-binding domains to a large number of substrates including cytoskeletal proteins, enzymes, and adaptor molecules (6–9). This stable interaction with substrates is important for the function of these kinases because of the relatively weak enzymatic activity and modest substrate specificity of their catalytic domains (9, 10); non-receptor tyrosine kinases lacking functional SH2 domains have dramatically reduced activity in vivo (11, 12). Src-substrate interaction leads to the phosphorylation of substrate tyrosine residues, thereby affecting a host of important cellular processes such as cell proliferation, adhesion, migration, differentiation, and survival (13, 14). Constitutively active Src mutants such as the v-Src gene product of Rous sarcoma virus induce malignant transformation, and activation of Src and other non-receptor tyrosine kinases has been observed in human tumors (15, 16).

Many substrates of normal and transforming tyrosine kinases have been identified, but in almost all cases the relative contribution of individual substrates to the biological activities elicited by these kinases remains unknown. This is because kinases generally phosphorylate many different substrates upon activation, and no experimental method is currently available to induce phosphorylation of a single substrate of interest in the absence of the simultaneous phosphorylation of others. Because this is a fundamental hurdle to understanding and manipulating tyrosine kinase-mediated signaling pathways, for example in validating targets for drug discovery downstream of tyrosine kinases, we sought to develop a method for inducing the phosphorylation of a single substrate in vivo.

We have described a general strategy, termed the functional interaction trap (FIT), for forcing the pairwise interaction of two proteins in vivo as a means to elucidate the functional consequences of that interaction (17). We reasoned that this approach could be adapted to induce specific substrate phosphorylation in vivo. Because the SH3 and SH2 domains of Src and other non-receptor tyrosine kinases are important for substrate binding and recognition, we felt it would be possible to replace the normal SH3 and SH2 domain-mediated interactions with an engineered, highly specific protein binding interface, thereby forcing a substrate of choice to bind the kinase and thus promoting its efficient phosphorylation. Because specificity would be conferred by the engineered binding interface, this approach would allow the experimental tyrosine phosphorylation of virtually any protein of choice in the cell, even when the kinase(s) that might normally phosphorylate that protein are not known.

We chose for the protein binding interface a pair of complementary synthetic amphipathic helices (coiled coils) originally selected by Plückthun and colleagues for high affinity heterodimerization in vivo, WinZipA and WinZipB (18) (hereafter referred to as ZipA and ZipB). We show here that the interaction between a ZipA-modified Src kinase and a ZipB-modified substrate leads to the specific tyrosine phosphorylation.
Stat3 cDNAs are of murine origin, p130Cas rat, and Cbl human, tag fused to the ZipB coiled-coil segment. The cortactin, paxillin, and derived vectors containing an N-terminal Myc tag alone or the Myc.

These fragments were ligated into the BamHI and NotI sites of pEBB-HA tag and the ZipA coiled-coil segment. The SH3 and SH2 domains were deleted from these constructs to eliminate stable interaction with physiological substrates. We also constructed plasmids expressing candidate Src substrates, fused at their N termini to the Myc epitope tag alone or to the Myc tag plus the ZipB coiled-coil segment. These included the known Src substrates Stat3, cortactin, paxillin, Cbl, and p130Cas (23–27). 293T cells were transfected with plasmids expressing each substrate, with or without the ZipB segment, in combination with the ZipA-tagged, SH3- and SH2-deleted Src construct. As shown in Fig. 1, specific phosphorylation of the ZipB-tagged substrates was observed, whereas the same substrates lacking the ZipB segment were not significantly phosphorylated under similar conditions. The tyrosine phosphorylation of substrates was demonstrated not only by immunoreactivity with antiphosphotyrosine antibodies but also by decreased gel mobility for the most highly phosphorylated forms (most prominent for paxillin and p130Cas). This experiment demonstrates that phosphorylation of substrates by the ZipA-modified Src kinase is dependent on the presence of the ZipB segment in the substrate.

**EVALUATING CONSEQUENCES OF TYROSINE PHOSPHORYLATION**

**RESULTS**

**Coiled-coil Domains Can Mediate Specific Interaction between Src and Substrates**—We initially constructed v-Src derivatives fused at their C termini to the HA epitope tag or to the HA tag and the ZipA coiled-coil segment. The SH3 and SH2 domains were deleted from these constructs to eliminate stable interaction with physiological substrates. We also constructed plasmids expressing candidate Src substrates, fused at their N termini to the Myc epitope tag alone or to the Myc tag plus the ZipB coiled-coil segment. These included the known Src substrates Stat3, cortactin, paxillin, Cbl, and p130Cas (23–27). 293T cells were transfected with plasmids expressing each substrate, with or without the ZipB segment, in combination with the ZipA-tagged, SH3- and SH2-deleted Src construct. As shown in Fig. 1, specific phosphorylation of the ZipB-tagged substrates was observed, whereas the same substrates lacking the ZipB segment were not significantly phosphorylated under similar conditions. The tyrosine phosphorylation of substrates was demonstrated not only by immunoreactivity with antiphosphotyrosine antibodies but also by decreased gel mobility for the most highly phosphorylated forms (most prominent for paxillin and p130Cas). This experiment demonstrates that phosphorylation of substrates by the ZipA-modified Src kinase is dependent on the presence of the ZipB segment in the substrate.
We could also demonstrate that phosphorylation of ZipB-containing substrates was dependent on the presence of ZipA in Src constructs lacking SH3 and SH2 domains. Myc- and ZipB-tagged substrates were expressed in 293T cells alone or together with SH3- and SH2-deleted Src constructs fused with either the HA tag alone or HA plus ZipA. Again, efficient substrate phosphorylation was evident only when the kinase and the substrate were fused to complementary coiled-coil segments (Fig. 2). In contrast, little if any tyrosine phosphorylation was observed in cells not expressing exogenous Src or expressing Src lacking the ZipA segment. Importantly, the specific increase in substrate phosphorylation was observed under conditions where little or no increase in phosphorylation of other cell proteins was apparent in whole cell lysates (see Fig. 2).
Figs. 1 and 2). Only if lysates were first immunoprecipitated with anti-Tyr(P) to concentrate tyrosine-phosphorylated proteins could a slight elevation in background phosphorylation be seen in cells expressing the modified kinase (Fig. 1, bottom). Taken together, these results demonstrate that the FIT approach using a coiled-coil binding interface could be used to induce the specific tyrosine phosphorylation of a substrate of interest in vivo in the absence of significant phosphorylation of other cell proteins. Similarly, specific FIT-mediated phosphorylation of substrates was also seen in transfected or retrovirally infected NIH-3T3 murine fibroblasts (data not shown).

**Effect of SH3 and SH2 Domains, Membrane Localization, and Position of the ZipA Segment on Substrate Phosphorylation by Src**—We expected that deletion of the SH3 and SH2 domains of Src would be essential to decrease background phosphorylation seen in cells expressing the modified kinase (Fig. 1, bottom). Taken together, these results demonstrate that the FIT approach using a coiled-coil binding interface could be used to induce the specific tyrosine phosphorylation of a substrate of interest in vivo in the absence of significant phosphorylation of other cell proteins. Similarly, specific FIT-mediated phosphorylation of substrates was also seen in transfected or retrovirally infected NIH-3T3 murine fibroblasts (data not shown).

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**Effect of SH3 and SH2 Domains, Membrane Localization, and Position of the ZipA Segment on Substrate Phosphorylation by Src**—We expected that deletion of the SH3 and SH2 domains of Src would be essential to decrease background (i.e., FIT-independent) phosphorylation of physiological substrates. To test this, we compared the activity of Src variants in which the SH3 and SH2 domains were either intact or deleted (see Fig. 3 for the structure of various Src derivatives). As expected, phosphorylation of p130Cas by Src constructs with intact SH3 and SH2 domains was very efficient, irrespective of the presence of coiled-coil segments in either the kinase or its substrate (Fig. 3, lanes 2, 3, 6, 7, 11, 12, 15, and 16). By contrast, for Src mutants lacking the SH3 and SH2 domains, efficient phosphorylation of p130Cas was only seen when both the substrate and kinase were fused to complementary coiled-coil segments (Fig. 3, lanes 14 and 18). This was the case even though the deleted Src constructs were expressed at considerably higher levels than the full-length versions (see Fig. 3, anti-HA panel). Similarly, FIT-independent phosphorylation of Stat3, cortactin, paxillin, and Cbl was also observed when SH3 and SH2 domains were present in Src (data not shown), although it was generally less extensive than in the case of p130Cas. Thus, deletion of the SH3 and
SH2 substrate-binding domains of the kinases is essential for the success of the FIT approach.

We also compared the activity of Src constructs where the HA epitope tag and coiled-coil segment were positioned at the N terminus in place of the normal N-myristoylation signal or at the C terminus, leaving the myristoylation signal intact. N-terminal myristoylation is required for transformation by v-Src, presumably because critical substrates are localized at the plasma membrane (28, 29). No qualitative difference was seen in FIT-induced phosphorylation of p130Cas depending on the presence of the myristoylation signal or on the location of the ZipA peptide (Fig. 3, lanes 12, 14, 16, and 18). Similar results were seen with other substrates (data not shown). Thus, the proximity of the substrate and kinase mediated by the coiled-coil interaction is sufficient to induce efficient substrate phosphorylation, and neither the position of the coiled-coil segment in the kinase nor the subcellular localization of the kinase appears to be critical for efficient FIT-induced phosphorylation of substrates. Taken together, these results indicate that the FIT strategy is robust and likely to be widely adaptable to different specific substrates and fusion proteins.

**FIT-mediated Phosphorylation Is Compatible with Downstream Signaling**—For the FIT approach to be useful in dissecting the functional consequences of substrate phosphorylation, it is important that FIT-induced phosphorylation have the same functional consequences as phosphorylation induced under normal physiological conditions. Potential problems with this strategy might include phosphorylation of physiologically irrelevant sites or inappropriate subcellular localization of phosphorylated substrates. Furthermore, coiled coil-mediated heterodimerization is relatively strong (K_d = 24 nM (18)), raising the possibility that interaction with the kinase could be so stable as to prevent efficient dissociation of the substrate and its subsequent relocalization. However, immunodepletion experiments indicated that the great majority of tyrosine-phosphorylated ZipB-tagged substrate was not stably associated with ZipA-tagged Src (Fig. 4), demonstrating that the coiled-coil interaction undergoes dissociation after substrate phosphorylation in vivo.

We utilized the Stat3 signaling pathway to address directly the issue of physiological relevance. In normal signaling, tyrosine phosphorylation of Stat3 induces the formation of SH2-mediated homodimers, which translocate to the cell nucleus where they recognize specific DNA elements and activate gene transcription, leading to physiological responses (30). Stat3 activation has been reported to be required for transformation by Src, and constitutive activation has been reported to be sufficient to induce transformation (23, 31, 32). To test whether FIT-induced phosphorylation could induce the same output as physiological phosphorylation, we transfected constructs expressing Src and Stat3 with or without complementary coiled coils along with a Stat3-dependent reporter construct driving luciferase expression (22). As shown in Fig. 5, luciferase activity was significantly increased by FIT-mediated phosphorylation of Stat3, but not when either the kinase or substrate lacked the coiled-coil segment. The degree of stimulation of Stat3-dependent transcription induced by FIT was similar to that obtained with wild-type v-Src under comparable conditions and a comparable expression level (Fig. 5). Thus, the FIT approach is compatible with downstream signaling, leads to phosphorylation of physiologically relevant sites, and can promote an appropriate physiological response.

**Elucidating the Functional Consequences of Tyrosine Phosphorylation for a Substrate of Choice**—p130Cas contains more than 10 tyrosine phosphorylation sites, and its phosphorylation has been implicated in cell motility, adhesion, and cell transformation (27, 33). Cells lacking p130Cas are resistant to transformation by Src (34). To address directly the specific consequences of p130Cas phosphorylation in cells, we expressed various Src constructs and p130Cas, with or without the complementary coiled coils, in NIH-3T3 fibroblasts. Only in cells expressing the combination of modified Src and p130Cas fused to complementary coiled coils was a clear difference from controls observed; a large percentage of transfected cells exhibited a distinct morphological alteration characterized by a bipolar, fusiform shape with long, thin terminal processes (Fig. 6). In multiple experiments, 20–25% of cells transfected with both ZipA-ΔSrc and ZipB-p130Cas exhibited this altered morphology, compared with 3–5% for all controls. This is consistent with previous reports showing that p130Cas localizes to focal adhesions and that its phosphorylation correlates with cell migration (27, 33). These mor-
phological changes were not observed when the ZipA-Src construct lacked catalytic activity (data not shown), so we can state with confidence that they were directly attributable to phosphorylation of p130Cas.

**DISCUSSION**

A Novel Strategy for Validating the Functional Significance of Post-translational Modifications—FIT was conceived as a general strategy to probe the functional consequences of interaction between two proteins (17). Here we show that this approach can be adapted to induce the efficient tyrosine phosphorylation in the cell of a single protein of choice, in the absence of significantly increased phosphorylation of other proteins. This approach is robust and likely to be widely applicable in a variety of contexts; we have also generated FIT-compatible variants of the Abl tyrosine kinase that are as effective as the Src constructs reported here (data not shown). The ability to induce specific phosphorylation of a candidate substrate or substrates will be invaluable in teasing out the functional consequences of kinase activation. Although genetic knockout and RNAi approaches can partially address which proteins are necessary for a particular biological response to activation of a kinase, FIT is designed to test which specific phosphorylations are sufficient to induce a biological response of interest. Previously this could only be addressed by expressing substrate mutants in which specific phosphorylatable residues had been changed to acidic residues; however, this approach requires a knowledge of specific phosphorylation sites, and mutation often does not accurately mimic the effects of phosphorylation, particularly for tyrosine-phosphorylated proteins.

There is now great interest in defining the global state of phosphorylation in the cell (the phosphoproteome) under various physiological states and in disease (2–5). The value of such information would be greatly enhanced, however, if there were methods to validate the functional significance of any phosphorylation that is observed. FIT could play an important role in such efforts. For example, FIT could be used to validate potential targets for drug discovery downstream of kinases whose activation affects human health, for example those involved in cancer, immune cell regulation, and angiogenesis. More broadly, we envision that the FIT strategy will also be useful for elucidating the functional consequences of other post-translational modifications mediated by enzymes that have relatively weak catalytic activity and thus use substrate-binding domains (analogous to the SH3 and SH2 do-

**FIG. 5. FIT-mediated tyrosine phosphorylation of Stat3 induces Stat3-dependent transcription.** 293T cells were transfected with a Stat3-dependent transcriptional reporter driving luciferase expression along with Stat3 expression constructs fused with Myc epitope (STAT3) or Myc epitope plus ZipB segment (ZipB-STAT3) with or without SH3- and SH2-deleted Src constructs fused with HA epitope (MyrΔSrc-HA) or HA epitope plus ZipA segment (MyrΔSrc-ZipA-HA). As a positive control, the amount of activity obtained when a comparable amount of full-length Myr-Src-HA (wild-type v-Src) was expressed is also shown. Data is expressed as mean ± S.E. of three observations and was analyzed by two-way analysis of variance. The asterisks indicate $p < 0.001$ as compared with controls; a significant increase in Stat3-dependent expression is observed only when both Src and Stat3 are fused to complementary coiled-coil segments.

**FIG. 6. FIT-mediated tyrosine phosphorylation of p130Cas by Src leads to altered morphology in NIH-3T3 cells.** NIH-3T3 murine fibroblasts were co-transfected with eGFP expression vector along with plasmids expressing Src and/or p130Cas. Dishes were observed under UV illumination to identify transfected (eGFP-expressing) cells, and photomicrographs were taken of representative fields 2 days post-transfection. The panels show the following: A, eGFP alone; B, HA-ΔSrc; C, HA-ZipA-ΔSrc; D, Myc-p130Cas; E, HA-ΔSrc + Myc-p130Cas; F, HA-ZipA-ΔSrc + Myc-p130Cas; G, Myc-ZipB-p130Cas; H, HA-ΔSrc + Myc-ZipB-p130Cas, and I, HA-ZipA-ΔSrc + Myc-ZipB-p130Cas. FIT-mediated tyrosine phosphorylation of p130Cas by Src in panel I results in an elongated, bipolar shape with thin terminal processes (arrowheads). Similar results were observed using myristoylated Src constructs (data not shown).
Evaluating Consequences of Tyrosine Phosphorylation

Because FIT replaces normal substrate-binding domains with an artificial binding interface, it could be argued that this may lead to the phosphorylation of physiologically inappropriate or irrelevant substrates; for example, FIT may force Src to phosphorylate substrates that are not normally expressed in the same cell as wild-type Src or in the same subcellular compartment. It is important to realize, however, that FIT is a tool to explore the consequences of phosphorylation; it is not intended to implicate the specific kinase used to induce phosphorylation as the one that mediates phosphorylation in the course of normal signaling. In fact this is experimentally advantageous as FIT-induced phosphorylation by Src is likely to mimic phosphorylation normally induced by a variety of tyrosine kinases. Other methods, such as the use of nucleotide analogs that specifically target a single, modified kinase (36–38), can then address the issue of which kinase normally induces a specific phosphorylation event in vivo.

Another important practical consideration for FIT experiments is the level of expression of the FIT-modified kinase and substrate. Obviously both the absolute extent of substrate phosphorylation and the percent phosphorylated will depend on these expression levels. The amount of expression in the experiments shown in Figs. 1–4 was chosen to emphasize the absolute extent of specific substrate phosphorylation (moderately low kinase and high substrate expression). At relatively higher levels of kinase expression, more nonspecific (FIT-independent) phosphorylation of both endogenous and overexpressed substrates begins to be observed (data not shown). Indeed, it has been shown previously that v-Src mutants with impaired or absent SH3 and SH2 domain function can in some cases induce malignant transformation when highly expressed (39, 40). Of course, for experiments designed to test the functional significance of tyrosine phosphorylation, it is likely to be advantageous to express both the substrate and kinase at relatively low levels comparable with endogenous levels of expression. Under these conditions, FIT-independent phosphorylation will be negligible.

In conclusion, we have shown that FIT can promote the pairwise association of a kinase and specific substrate and the subsequent phosphorylation of that substrate. Thus, it is now possible to assess the biological effects resulting from phosphorylation of a particular substrate in vivo. FIT can serve as an important validation and discovery tool for proteomic analysis that complements other methods currently available.

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mains of Src) to direct modification of specific targets. Obviously candidates would include serine-threonine kinases such as mitogen-activated protein kinases and cyclin-directed kinases, histone-modifying enzymes, proline isomerases, and many others. Of course, the FIT approach might not be appropriate for those enzymes for which the modular substrate-binding domains are also necessary for catalytic activity itself.

Perhaps the most exciting potential use of the FIT approach will be as the basis for cell-based proteomic screens for novel, functionally relevant interactions or post-translational modifications. In the near future when expression libraries of full-length cDNAs (35) fused to coiled-coil segments are available, it will be possible to perform proteome-wide screens to identify those proteins whose interaction with, or modification by, a protein of choice leads to a biological output of interest. Such screens would not require any preconceived ideas about the identity of potential binding partners or substrates. For example, this approach could be used to screen for proteins whose tyrosine phosphorylation leads to changes in proliferation, adhesion, or motility; such proteins would likely include novel targets for intervention in disease. Although it is possible that such screens would miss some functionally important modifications due to technical reasons (fusion of a substrate to the coiled-coil segment might affect its biological activity, for example), any positive result would provide strong evidence that modification of the substrate plays a functional role in the biological activity of interest. Such functional screens would complement and enrich the descriptive and correlative data on protein modifications that are emerging from standard proteomic analyses.

Experimental Considerations—Several implications of the FIT approach deserve comment. First, the strong dependence of substrate phosphorylation on FIT-mediated protein-protein interaction for Src (or Abl) constructs lacking SH3 and SH2 protein-binding domains highlights the important role normally played by these domains. Clearly the catalytic domains of non-receptor tyrosine kinases lack the inherent specificity to drive efficient substrate phosphorylation in vivo in the absence of stable binding mediated by the SH3 and/or SH2 domains. Somewhat surprisingly, we found that the extent of substrate phosphorylation was apparently insensitive to the location of the ZipA segment in Src. As ZipA and ZipB associate in a parallel (head-to-head) fashion (18), this strongly suggests that there is considerable conformational flexibility in the kinase-substrate interaction and that substrate phosphorylation does not depend on a precise orientation relative to the catalytic domain.

Because FIT replaces normal substrate-binding domains with an artificial binding interface, it could be argued that this may lead to the phosphorylation of physiologically inappropriate or irrelevant substrates; for example, FIT may force Src to phosphorylate substrates that are not normally expressed in the same cell as wild-type Src or in the same subcellular compartment. It is important to realize, however, that FIT is a tool to explore the consequences of phosphorylation; it is not intended to implicate the specific kinase used to induce phosphorylation as the one that mediates phosphorylation in the course of normal signaling. In fact this is experimentally advantageous as FIT-induced phosphorylation by Src is likely to mimic phosphorylation normally induced by a variety of tyrosine kinases. Other methods, such as the use of nucleotide analogs that specifically target a single, modified kinase (36–38), can then address the issue of which kinase normally induces a specific phosphorylation event in vivo.

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