Mapping of Synergistic Components of Weakly Interacting Protein-Protein Motifs Using Arrays of Paired Peptides*

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Protein-protein recognition usually involves multiple interactions among different motifs that are scattered over protein surfaces. To identify such weak interactions, we have developed a novel double peptide synthesis (DS) method. This method allows us to map protein-protein interactions that involve two linear discontinuous components from a polypeptide by the use of spatially addressable synergistic pairs of synthetic peptides. The DS procedure is based on the “SPOT” membrane-bound peptide synthesis technique, but to synthesize a mixture of two peptides, it uses both Fmoc (N-(9-fluorenyl)methoxycarbonyl)-alanine and Alloc-alanine at the first cycle. This allows their selective deprotection by either piperidine or tributyltin/palladium treatment, respectively. Using SPOT DS, we confirmed as a proof of principle that Elk-1 Ser383 phosphorylation by ERK-2 kinase is stimulated by the presence of the Elk-1-docking domain. SPOT DS can also be used to dissect protein-protein interactions that define phosphatase substrate affinity. Using this technique, we identified three new regions in the insulin receptor that stimulate the dephosphorylation of the receptor by protein-tyrosine phosphatase (PTP) 1B and presumably increase the selectivity of PTP for this substrate. These data demonstrate that the SPOT DS technique allows the identification of non-linear weakly interacting protein motifs, which are an important determinant of protein kinase and phosphatase substrate specificity and of protein-protein interactions in general.

The substrate specificity of kinases and phosphatases is often determined by multiple domain-domain interactions (1). An example is the Src homology domain of the Src family of kinases and also found in tyrosine phosphatases SHP1 and 2, which interacts with phosphotyrosine residues. The study of these domain-domain interactions often involves co-immunoprecipitation or yeast two-hybrid-like approaches. Co-immunoprecipitation often fails to detect weak interactions, whereas two-hybrid-like approaches are time-consuming, usually require nuclear import of the proteins under study, and are prone to interference by host proteins. The SPOT technique, which involves direct on-membrane peptide microsynthesis, is another powerful tool to study protein-protein interactions in the case of linear motifs (2, 3). Although SPOT allows the testing of a very large number of different peptide motifs for interaction with a given protein, it can only detect relatively strong stable protein-domain interactions, e.g. in epitope mapping (4). To expand this technology so that weak protein-protein interactions can be detected as well, we have modified the SPOT protocol. Instead of synthesizing one peptide per spot, we have set up a procedure that allows the synthesis of two different peptides per spot. This can be used to examine temporary/weak interactions between an enzyme and its substrate. On each spot, one peptide corresponds to a sequence of the substrate that is stably modified by the enzyme, whereas the second peptide reveals a second interaction that occurs between the enzyme and the substrate sequence assayed (see Fig. 2B). This setup is reminiscent of yeast two-hybrid systems where weak protein-protein interactions trigger transcriptional activation but has the advantage that many different peptides (hundreds) can be studied on a single membrane.

To validate the SPOT double synthesis (SPOT DS) approach, we have focused as proof of principle on the extracellular signal-regulated kinase ERK-2, a mitogen-activated protein kinase. One of the substrates of ERK is Elk-1 Ser383 (5). Previous work has established that in order for ERK-2 to efficiently phosphorylate its substrate, an interaction with a second linear motif, the docking domain that is present in Elk-1, is necessary (reviewed in Ref. 6). Another binding motif in Elk-1 named FXFP is also involved in the ERK-2-Elk-1 complex formation (7). Although a short peptide from the Elk-1-docking domain is by itself not sufficient to efficiently bind ERK-2 on SPOT, we show here using SPOT DS that the additional presence of the docking domain in the vicinity of the Elk-1 Ser-containing phosphorylation site increases Ser phosphorylation. This result suggests that the SPOT DS is a sensitive approach to detect and define weak synergistic protein-protein interaction motifs.

In a second example, we have studied a protein-tyrosine phosphatase for its substrate recognition requirements. PTP-1B is critically involved in insulin receptor dephosphorylation in vivo (8). PTP-1B dephosphorylates the insulin receptor kinase at its major autophosphorylation sites Tyr1158, Tyr1162, and Tyr1163 with the greatest efficiency for the latter two (9). Apart from the phosphotyrosine-dependent interaction be-

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1 The abbreviations used are: SPOT DS, SPOT double synthesis; ERK, extracellular signal-regulated kinase; Fmoc, N-(9-fluorenyl)methoxycarbonyl; Alloc, N-allyloxycarbonyl; PBS, phosphate-buffered saline; PTP, protein-tyrosine phosphatase; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; YAP, Yes-associated protein; WW1, domain characterized by tryptophanes.
tween the insulin receptor and the catalytic domain of PTP-1B, another region of PTP-1B involving PTP-1B Tyr152 and Tyr153 is also able to directly interact with the insulin receptor in a non-phosphorylation-dependent manner (10), although the critical domains in the insulin receptor have not yet been defined. Using dephosphorylation of the insulin receptor-auto-phosphorylated tyrosines as readout, we identified additional insulin receptor-derived peptides that enhanced PTP-1B activity. These data illustrate the use of SPOT DS for phosphatases as well as for kinase substrate definition studies.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The pGEX-4TK vector corresponds to pGEX-2TK (Amersham Biosciences) with multicloning sites from the pGEX-4T3 vector (Amersham Biosciences). pGEX-4TK-3-PTP-1B D181A encodes the GST-human PTP-1B fusion protein from amino acids 1 to 289 with Ali18 instead of an Asp (11). pGEX-4TK-3-PTP-1B full-length corresponds to the entire human cDNA (GenBank accession number M31724). pGEX-2TK-YAP WW1 encodes the human cDNA region (nucleotides 429 to 997) corresponding to the YAP WW1 domain (amino acids 162–217) (12). GST-EKR-2 and GST-MEK-EE (S218E, S222E) were kindly provided by Montserrat Camps (13, 14).

**GST Production and Protein Labeling**—Transformed bacteria were grown at 37 °C in LB medium in the presence of ampicillin (0.1 mg/ml). For the induction of GST constructs, isopropyl-1-thio-

**Kinase Assays and Western Blot**—SPOT membranes were blocked in PBS, 0.1% Tween 20, and 0.5 mM dithiothreitol. GST fusion proteins were eluted (see above) and added to blocked SPOT membranes in kinase buffer II. After 40 min at 37 °C under agitation, blots were extensively rinsed.

**Dephosphorylation Studies**—SPOT membranes were blocked 2 h in dephosphorylation buffer (Western wash buffer, 1× SPOT blocking buffer, and 1 mM dithiorthretiol). GST was cleaved off by thrombin on glutathione beads in PBS. Wild type PTP-1B full-length protein (10 μg) was incubated with the SPOT membranes in dephosphorylation buffer at 37 °C for 6 h. GST-PTP-1B was removed by extensive washing in PBS, 0.1% Tween 20 (16). Dephosphorylation was monitored by ECL (see above for conditions) using an anti-phosphotyrosine antibody (4G10, 1,3,000 dilution, Upstate Biotechnology).

**In silico Receptor Modeling**—The insulin receptor surface accessibility model was produced using published coordinates (17). The Connolly surface was calculated with a probe radius of 1.4 Å using InsightII (Accelrys) software.

**Cellular Assays**—The wild type insulin receptor expression vector contained the 4.4 kilobase pairs of full-length human insulin receptor cDNA cloned into pRC-CMV2, vec2. The exchange of the two lysine codons into two alanines to make Mut1 and Mut3 was made using Stratagene QuikChange kit. The plasmid (100 ng) was PCR-amplified with Pfu polymerase (Promega) using an extension time relative to the size of the plasmid used (0.5 kilobase pairs/min) for 14 cycles. The wild type plasmid was digested with DpnI for 1 h at 37 °C. Ultracompotent cells (XL2-Blue, Stratagene) were transformed and plated on selective medium. For each mutation, a pair of primers was designed that carried the 3′ end of the interior arm of the plasmid.
the codon changes in the center of the sequence (underlined sequences): 5′-CGG GAT GGC C TAC CTG AAC GCC GCG GCG TTT GT-3′ and 5′-G ATG CAC AAA CGC CGC GGC GTT CAG GTA GG-3′ for Lys1153-Lys1154 and 5′-AC GGA GGC GCG GCA AAC GGG CGG ATT CTG ACC-3′ and 5′-CCC GTT TGC CGC GCC TCC GTT CAT GTG TGT GTA AG-3′ for Lys1368-Lys1369. All of the constructs were checked by sequencing analysis.

Human embryonic kidney 293 cells were plated in 24-well plates at 5 × 10⁴ cells/well and transfected with 0.5 μg of DNA using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) and a ratio of 2.5 μl of reagent for 1 μg of DNA. One-tenth of a reporter plasmid was co-transfected to monitor the efficiency of transfection. Twenty-four hours posttransfection, cells were starved for at least 4 h in serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) and incubated with 100 nM bovine insulin (Sigma). After 10 min of stimulation, the insulin was removed by replacement with fresh medium. The receptor dephosphorylation process was stopped after 10 min by a rapid removal of the serum-free medium and the addition of protein sample buffer with phosphatase and kinase inhibitors.

Western blot detection was with rabbit polyclonal anti-phospho-Insulin receptor/IGF insulin receptor (Tyr 1162-Tyr1163) and a mouse monoclonal antibody against human insulin receptor β-subunit (CT-3), both from BIOSOURCE.

RESULTS

SPOT Double Synthesis: Proof of Feasibility—To synthesize two different peptide sequences on the same spot, an equimolar mixture of Fmoc-Ala and Alloc-Ala was incorporated at the first cycle on the membrane (Fig. 1A). At the end of this cycle, piperidine was used to specifically remove the Fmoc moiety, leaving the other half of the Ala residues blocked for extension by the Alloc moiety. The first peptide synthesis was performed...
using standard Fmoc chemistry. At the end of this synthesis, amino groups from the elongated peptide were permanently blocked using acetic acid anhydride. Alloc groups were removed by palladium-catalyzed hydrostannolytic cleavage in the presence of tributyltin hydride (15). Finally, the second peptide was synthesized using standard Fmoc chemistry. We confirmed that the Alloc deprotection had been successful by staining with bromphenol blue, an indicator for the freshly deprotected amino groups (4). To determine whether the entire process had been successful, we synthesized two different peptides together or separately: IYETDYZRKGG (the autophosphorylation site of the human insulin receptor; Z stands for phosphotyrosine) and YPPYPPPPYPS (from the p53-binding protein-2, p53BP-2), which are binding motifs for the PTP-1B D181A substrate-trapping mutant (9, 16) and the YAP WW1 domain (18), respectively. As shown in Fig. 1B, GST-PTP-1B D181A specifically recognized spots that contained the insulin receptor peptide and the GST-YAP WW1 domain bound those with p53BP-2. The spots, which corresponded to double synthesis (Fig. 1B, top), were bound by both probes. As a control, we confirmed that radiolabeled GST alone did not bind any of the spots (data not shown). These data indicate that co-synthesis of two different peptide sequences on the same SPOT had been successful.

**Detecting Weak Protein-Protein Interactions Using SPOT DS**—To test whether the SPOT DS approach can reveal weak synergistic binding motifs, we tested a sequence known to be important in protein-protein interactions, the docking domain of Elk-1. ERK-2 interacts via its carboxyl common docking domain with this Elk-1-docking domain, which permits the efficient phosphorylation of Ser383 of Elk located in a different domain (Fig. 2A) (5). ERK-2, activated or not by MEK, does not stably bind to spots that contain either only the Elk-1-docking domain (KGRKPRDLELP) or the Elk-1 phosphorylation site (FWSTLSPIAPR) (data not shown). Therefore, this finding confirms that the ERK-2/Elk1-phosphorylation site interaction is weak.

Fig. 2B illustrates the rationale of the assay. Double syntheses on membranes were performed as follows: the Elk-1 phosphorylation site was first synthesized on three spots followed by either the synthesis of the Elk-1-docking domain peptide, an unrelated sequence, or no second peptide on the same spots. SPOT DS membranes were then incubated in kinase buffer II with GST-MEK-EE (constitutively activated), GST-ERK-2 alone, or both together (at a ratio of 1:5 w/w). The MEK protein was required to activate ERK-2 (See Fig. 2A for the MEK-Elk-1 cascade). As shown in Fig. 2A (bottom), limited phosphorylation of Ser383 was seen when the Elk-1 phosphorylation site was presented alone or in association with the control negative peptide, but serine phosphorylation was strongly enhanced when associated with the Elk-1-docking domain peptide. As

| Spot | Sequence |
|------|----------|
| 43   | ADGMAYLNALK |
| 44   | AYNLKFFVHR |
| 45   | AKTPVHRDLAA |
| 54   | DYSRRGKGKGL |
| 55   | KGRGGLPVRW |
| 57   | PYTHMNNGKKR |
| 98   | MNGGKRGRIL |
| 99   | KKXGRXLT |

**Consensus**

**KKxGRxL**

**Table 1.** Insulin receptor peptides that interact with PTP1B

Conserved amino acids are highlighted in black. Underlined amino acids are those shared between the two interacting insulin receptor domains 54–55 and 98–99.
expected, GST alone did not interact with any of these spots (data not shown). We conclude that the increased in serine phosphorylation is because of a local increase of ERK-2 concentration resulting from the presence of the docking domain. This enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the expected, GST alone did not interact with any of these spots (data not shown). We conclude that the increased in serine phosphorylation is because of a local increase of ERK-2 concentration resulting from the presence of the docking domain. This enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the expected, GST alone did not interact with any of these spots (data not shown). We conclude that the increased in serine phosphorylation is because of a local increase of ERK-2 concentration resulting from the presence of the docking domain. This enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the expected, GST alone did not interact with any of these spots (data not shown). We conclude that the increased in serine phosphorylation is because of a local increase of ERK-2 concentration resulting from the presence of the docking domain. This enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the expected, GST alone did not interact with any of these spots (data not shown). We conclude that the increased in serine phosphorylation is because of a local increase of ERK-2 concentration resulting from the presence of the docking domain. This enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the enhanced activity was most prominent when ERK-2 had been activated by MEK-EE, but ERK-2 alone also displayed the
receptor in cells, stimulate them with insulin, and examine the phosphorylation status of the receptor following insulin withdrawal. Our assumption was that dephosphorylation of the insulin receptor would depend on PTP-1B as has been demonstrated in PTP-1B knock-out mice (8, 20). Because the second PTP-1B binding motif is directly C-terminal of the autophosphorylation site, we considered it likely that mutating this sequence would (also) affect insulin receptor autophosphorylation efficiency; therefore, we decided to test insulin receptor mutated for the first and third peptide motifs named Mut1 and Mut3 (see Fig. 5A, bottom). In both motifs, the two lysine codons were mutated into alanines. As shown in Fig. 5A, top, insulin receptor Mut3 shows increased levels of phosphorylation as compared with wild type or insulin receptor Mut1 following insulin stimulation of the cells. This finding suggests that at least the C-terminal peptide is required for efficient insulin receptor dephosphorylation in a cellular context. However, interpretation of this experiment is not straightforward. It would be necessary to demonstrate that the insulin receptor kinase activity is not affected by the mutations as may be the case for Mut1. It is also not certain that intracellular dephosphorylation of the overexpressed insulin receptor depends on PTP-1B alone. Further evidence for a direct interaction between PTP-1B and these insulin receptor motifs requires further structural and enzymatic studies and ideally co-crystallization.

**DISCUSSION**

We have shown that it is technically feasible to adapt the SPOT procedure to synthesize mixtures of peptides. This novel procedure is useful for the identification of domains whose weak interaction is critical in protein-protein interactions that are the basis of enzymatic kinase and phosphatase reactions. In contrast to other procedures, the SPOT technique is restricted to testing peptide motifs rather than large proteins. On the other hand, the SPOT provides a convenient approach to study modified peptides such as phosphorylated, glycosylated, or methylated derivatives. The procedure has been automated and adaptable robots are commercially available so that hundreds of syntheses can be performed in a single run. Hitherto, the fine mapping of protein-protein interactions would require the co-crystallization of the enzyme plus its full-sized substrate or the construction and study of large numbers of mutants. The identification of protein regions that are required for optimal enzymatic recognition may result in the development of highly selective peptidomeric inhibitors that do not target the usually conserved catalytic domain of the enzyme.

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