14-3-3 ζ interacts with the α -chain of human interleukin 9 receptor

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Interleukin 9 (IL-9) exerts its pleiotropic effects through the IL-9 receptor (IL-9R) complex, which consists of the IL-9R α -chain, which determines the cytokine specificity, and the IL-2 receptor γ -chain. In the present study we used a modified yeast two-hybrid system to isolate cDNA species encoding proteins that interacted with the intracellular domain of the human IL-9R α -chain (hIL-9R α). We have identified 14-3-3 ζ as an hIL-9R α -interacting protein. We also mapped residues 518–522 (Arg-Ser⁵¹⁹-Trp-Thr⁵²¹-Phe) in hIL-9R α and helix I of 14-3-3 ζ as being

important for interaction. Moreover, peptide competition experiments suggested that interaction between hIL-9R α and 14-3-3 ζ requires the phosphorylation of Ser⁵¹⁹ or Thr⁵²¹. This is the first demonstration that 14-3-3 can interact with a non-tyrosine kinase receptor. The interaction between 14-3-3 and IL-9R α but not IL-4R α also suggests a potential role for 14-3-3 in determining cytokine specificity.

Key words: binding motif, cytokine, protein–protein interaction.

INTRODUCTION

Interleukin 9 (IL-9) is a multifunctional T-cell-derived cytokine with a variety of biological activities including the proliferation of activated and transformed T-cells [1,2], the production of immunoglobulins by B-cells [3] and the proliferation and differentiation of erythroid and myeloid precursors [4,5]. In addition, the involvement of IL-9 in both human and murine tumorigenesis has been suggested [6,7].

IL-9 exerts its biological effects as a result of binding to the IL-9 receptor (IL-9R), which belongs to the IL-2 receptor γ -chain superfamily [8]. This family includes receptors for IL-2, IL-4, IL-7, IL-9 and IL-15; all these receptors have a common IL-2 receptor γ -chain and at least one ligand-specific subunit [8]. In addition to structural similarities, these receptors also share certain signal transduction pathways, such as activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [8]. It has been shown that IL-9 stimulates the activation of JAK kinases, STAT1, STAT3 and STAT5, the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 and the expression of nuclear proto-oncogenes [9–12]. Specific cytoplasmic domains of the human IL-9R α -chain (hIL-9R α) important for cell proliferation, anti-apoptotic activity, growth regulation, STAT and IRS activation have been determined [11,12].

14-3-3 proteins are a family of highly conserved acidic proteins that are involved in mitogenic signalling, cell cycle control, apoptosis, cell adhesion and nuclear localization [13–17]. 14-3-3 proteins can form homodimers or heterodimers, which allow them to function as an adapter, linker, scaffold or co-ordinator in assembling signalling complexes [18]. Crystallographic studies have indicated that each monomer in a 14-3-3 dimer contains a large groove that can dock with other molecules [19,20]. 14-3-3

proteins interact with a number of signalling proteins, including Raf, Bcr and Bcr/Abl, polyomavirus middle T antigen, phosphoinositide 3-kinase, cdc25 phosphatase, PTPH1 phosphatase, the proto-oncogene product Cbl, tryptophan hydroxylase, protein kinase C and IRS-1 [21–28]. In addition, insulin-like growth factor I receptor, adhesion receptor platelet glycoprotein Ib α and glucocorticoid receptor have been shown to interact with 14-3-3 proteins [29,30].

To identify molecules involved in IL-9R signalling, we performed yeast two-hybrid screening to identify proteins binding to the intracellular domain of hIL-9R α . Several interacting clones encoding 14-3-3 isoforms with the highest sequence similarity to 14-3-3 ζ were identified. The interaction between full-length 14-3-3 ζ and hIL-9R α was confirmed by co-immunoprecipitation, and the interacting domain of hIL-9R α was identified through a yeast two-hybrid assay. We also showed that serine or threonine phosphorylation is necessary for the binding of 14-3-3 ζ to hIL-9R α and identified a novel motif in hIL-9R α for binding 14-3-3 ζ .

EXPERIMENTAL

Reagents and cell culture

Polyclonal antibodies for IRS-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-phosphotyrosine and anti-(c-Myc) (9E10) antibodies were from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. Monoclonal anti-(hIL-9R α) and anti-FLAG M2 (FLAG is Asp-Tyr-Lys-Asp-Asp-Asp-Lys) antibodies were purchased from R & D Systems (Minneapolis, MN, U.S.A.) and Eastman

Abbreviations used: FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Lys; hIL-9R α , human IL-9R α -chain; IL, interleukin; IL-9R, IL-9 receptor; IRS, insulin receptor substrate; JAK, Janus kinase; MBP, maltose-binding protein; STAT, signal transducer and activator of transcription.

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Figure 1 14-3-3 ζ associates with IL-9R but not with IL-4R

Upper panel: 293 cells were co-transfected with 5 μ g of FLAG-epitope-tagged hIL-9R α (pFlagIL-9Rwt) and 5 μ g of Myc-epitope-tagged 14-3-3 ζ (pMTmyc14-3-3 ζ) expression vectors. Cell lysates were immunoprecipitated (IP) with anti-FLAG (lanes 2, 4, 6 and 8) and control rabbit IgG (lanes 1, 3, 5 and 7) antibodies respectively. Immunoprecipitates were subjected to SDS/PAGE and immunoblotted (WB) with anti-(hIL-9R α) (lanes 1–4) or anti-Myc (lanes 5–8) antibodies. Lower panel: 293 cells were co-transfected with 5 μ g of FLAG-epitope-tagged mIL-4R α (pFlagIL-4R) and 5 μ g of Myc-epitope-tagged 14-3-3 ζ (pMTmyc14-3-3 ζ) expression vectors. Cell lysates were immunoprecipitated with control rabbit IgG (lanes 1 and 4), anti-FLAG (lanes 2 and 5) or anti-Myc (lanes 3 and 6) antibodies. Immunoprecipitates were subjected to SDS/PAGE and immunoblotted with anti-FLAG (lanes 1–3) or anti-Myc (lanes 4–6) antibodies. The positions of molecular mass markers are indicated at the right in both panels.

Kodak Company (Rochester, NY, U.S.A.) respectively. Cells of the 293 line were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, U.S.A.) in the presence

of 10% (v/v) fetal calf serum (HyClone, Logan, UT, U.S.A.) and 50 i.u./ml penicillin and 50 i.u./ml streptomycin (Gibco BRL).

Yeast two-hybrid screening

Yeast two-hybrid screening of a cDNA library derived from the murine lymphohaemopoietic progenitor stem-cell-factor-dependent cell line EMLC1 [31] was performed with a bait containing LexA-IL-9R α intracellular domain and JAK1 kinase domain (pBTM-JAK-IL9R), as described previously [32]. β -Galactosidase assays in solution and the calculation of β -galactosidase activity by the Miller method were as described previously [33,34].

Plasmid construction

The construction and characterization of pBTM-JAK-IL9R, pBTM-IL9R411, pBTM-IL9R441, pBTM-IL9R452, pBTM-IL9R473 and pBTM-IL9R522 have been described previously [32]. Other plasmids used for the yeast two-hybrid assay were prepared by subcloning PCR-amplified truncated IL-9R α intracellular domains into *EcoRI*/*Bam*HI sites of pBTM116 to generate pBTM-IL9R509 (residues 292–509), pBTM-IL9R513 (residues 292–513), pBTM-IL9R517 (residues 292–517) and pBTM-IL9RS519A (residues 292–522, in which Ser⁵¹⁹ was mutated to alanine).

The construction and characterization of pFlagIL-9Rwt has been described previously [12]. pFlag-mIL-4R was obtained from the Genetics Institute (Cambridge, MA, U.S.A.). pVP16-14-3-3 ζ was prepared by subcloning 14-3-3 ζ cDNA from pMTmyc14-3-3 ζ (a gift from Dr. J. Avruch, Harvard Medical School, Boston, MA, U.S.A.) into the *NotI* site of pVP16 vector. Cloning, preparation of fusion proteins with maltose-binding protein (MBP) of 14-3-3 ζ constructs and their coupling to the Sepharose beads have been described previously [35].

Transient cell transfection, immunoprecipitation and Western blot analysis

Cells (2×10^6) of the 293 line were transfected by the calcium phosphate precipitation method [36] with various plasmid combinations as indicated. After 48 h, cell lysates were prepared and immunoprecipitation and Western blot analysis were performed as described previously [32].

Peptide competition experiments

Non-phosphorylated peptides and peptides containing phosphoserine and phosphothreonine were purchased from BBF, IUPUI (Indianapolis, IN, U.S.A.). The peptide sequences were MLLPSVLSKARSWTF, MLLPSVLSKAR[pS]WTF ([pS] corresponds to pSer⁵¹⁹ in hIL-9R α) and MLLPSVLSKARSW[pT]F ([pT] corresponds to pThr⁵²¹ in hIL-9R α) respectively. Peptides were purified by HPLC; their molecular masses were confirmed by MS. For peptide competition experiments, lysates from 293 cells overexpressing hIL-9R α were preincubated with one of these peptides (1 mM) for 1 h, followed by the addition of lysates from 293 cells overexpressing 14-3-3 ζ . After a 1 h incubation at 4 °C, lysates were immunoprecipitated with anti-FLAG antibodies and Western blot analysis was performed as described above.

Binding of hIL-9R α to 14-3-3 ζ -conjugated beads

Lysates from 293 cells overexpressing hIL-9R α were incubated with 25 μ l of 50% (v/v) MBP-conjugated control beads or 14-3-3 ζ -conjugated beads for 2 h at 4 °C; the binding of hIL-9R α to 14-3-3 ζ was detected by Western blot analysis as described above.

RESULTS

Detection of proteins binding to the intracellular domain of hIL-9R α

To identify proteins able to bind to the intracellular domain of the hIL-9R α , we employed a modified yeast two-hybrid system. The LexA fusion vector pBTM116 was modified to include the expression of a kinase domain from JAK1 kinase that could phosphorylate the LexA fusion protein pBTM-JAK-IL9R and/or expressed proteins from a lymphohaemopoietic progenitor stem-cell-factor-dependent EMLC1 cDNA library [31]. Screening of 1.28×10^7 transformants resulted in 10 transformants that activated *HIS3* and *lacZ* reporter genes with pBTM-JAK-IL9R but not with pBTM-lamin. Eight of the clones encoded 14-3-3 proteins with the highest similarity to 14-3-3 ζ . To confirm that the interaction between the intracellular domain of hIL-9R α and 14-3-3 ζ was phosphoprotein-dependent, we performed a yeast two-hybrid assay with the use of bait either with (pBTM-JAK-IL9R) or without (pBTM-IL9R522) the JAK1 kinase domain. Phosphorylation caused by overexpression of JAK1 kinase approximately doubled the interaction between the intracellular domain of hIL-9R α and 14-3-3 ζ (results not shown). Further studies supported the notion that serine/threonine phosphorylation of IL-9R α is important for the interaction between IL-9R α and 14-3-3 ζ (see below).

Interaction of the intracellular domain of hIL-9R α , mIL-4R α and 14-3-3 ζ

To determine whether hIL-9R α associates with 14-3-3 ζ , 293 cells were co-transfected with a FLAG-epitope-tagged hIL-9R α expression vector (pFlagIL-9Rwt), and a Myc-epitope-tagged 14-3-3 ζ expression vector (pMTmyc14-3-3 ζ). After 48 h, cell lysates were prepared and immunoprecipitated with anti-FLAG monoclonal antibody or control antibody. Immunoblot analysis with anti-(hIL-9R α), anti-FLAG and anti-Myc antibodies showed that hIL-9R α associates with 14-3-3 ζ (Figure 1, upper panel, and results not shown for immunoblot with anti-FLAG). To test whether the binding of 14-3-3 ζ was specific for hIL-9R α , we co-transfected 293 cells with a FLAG-epitope-tagged mIL-4R α expression vector and a Myc-epitope-tagged 14-3-3 ζ expression vector. Previous studies have shown that intracellular domains critical for signal transduction are highly conserved between mouse and human IL-4R α [37]. Cell lysates were immunoprecipitated with control, anti-FLAG and anti-Myc monoclonal antibodies respectively. Immunoblot analysis with anti-FLAG and anti-Myc antibodies showed that, although mIL-4R α and 14-3-3 ζ were overexpressed in 293 cells, they did not co-immunoprecipitate (Figure 1, lower panel).

C-terminal domain of hIL-9R α contains a new 14-3-3 ζ -binding site

In an attempt to identify the minimum amino acid residues of hIL-9R α that are sufficient to interact with 14-3-3 ζ , we constructed various mutants with truncated C-terminal domains of hIL-9R α into pBTM116 and tested for their interactions with pVP16-14-3-3 ζ (Figure 2). pBTM-IL9R509, pBTM-IL9R513

Figure 2 14-3-3 ζ binds to a specific motif in the C-terminus of the hIL-9R α in the yeast two-hybrid assay

pBTM-LexA constructs with the full-length, truncated or mutated intracellular domain of hIL-9R α (pBTM-IL9R522, pBTM-IL9R517, pBTM-IL9R513, pBTM-IL9R509, pBTM-IL9R473, pBTM-IL9R452, pBTM-IL9R441, pBTM-IL9R411 and pBTM-IL9RS519A) were co-expressed with pVP16-14-3-3 ζ plasmid in L40 yeast. Transformants were assayed for β -galactosidase activity by the solution assay. β -Galactosidase activity is reported in Miller units (means $\pm$ S.D.) for a minimum of three independent colonies.

Protein	Binding site
IL-9R (518-522)	R-S -W-T-F
c-Raf-1 (256-261)	R-S -T- S -T-P
A-Raf (211-216)	R-S -T- S -T-P
c-Raf-1 (618-623)	R-S -A- S -E-P
A-Raf (579-584)	R-S -A- S -E-P
Cdc25b (306-311)	R-S -P- S -M-P
Cdc25b (358-363)	R-S -K- S -M-P
Cdc25c (213-218)	R-S -P- S -M-P
mT (273-278)	R-S -H- S -Y-P
A20 (562-567)	R-S -K- S -D-P
BAD (109-114)	R-S -H- S -Y-P
BAD (133-138)	R-S -R- S -A-P
IRS-1 (971-977)	R-P-T- R-S -V-P
PKC-γ (141-147)	R-C-V- R-S -V-P

Figure 3 hIL-9Rα contains a novel binding motif for 14-3-3 proteins

An alignment of the C-terminal region of hIL-9Rα (residues 518–522) with known 14-3-3-binding sites is shown. Bold letters indicate conserved amino acids, bold and italic letters indicate phosphorylated serine.

and pBTM-IL9R517 constructs were of particular interest because they contain a serine-rich motif that is similar to a previously identified 14-3-3-binding site. None of the hIL-9Rα constructs described above showed any activation of β-galactosidase activity in the yeast two-hybrid assay with 14-3-3ζ (Figure 2). This suggested that the C-terminal five residues of the hIL-9Rα intracellular domain (Arg-Ser-Trp-Thr-Phe) are critical for the binding of 14-3-3ζ to hIL-9Rα. To test whether Ser⁵¹⁹ in hIL-9Rα was crucial for binding 14-3-3ζ, we prepared construct pBTM-IL9RS519A containing full-length hIL-9Rα intracellular domain (residues 292–522), in which Ser⁵¹⁹ was mutated to Ala (Figure 2) and tested it in the yeast two-hybrid assay. As shown in Figure 2, a point mutation in the Arg-Ser-Trp-Thr-Phe motif into Arg-Ala-Trp-Thr-Phe in hIL-9Rα abolished the binding between hIL-9Rα and 14-3-3ζ. Comparison between the C-terminal domain (residues 518–522) of hIL-9Rα and other previously characterized 14-3-3-binding regions suggests a novel specific recognition motif in hIL-9Rα for binding 14-3-3 (Figure 3).

Phosphorylation of Ser⁵¹⁹ or Thr⁵²¹ is essential for the binding of hIL-9Rα to 14-3-3ζ

Because the results presented above indicated that Ser⁵¹⁹ in hIL-9Rα was important for binding 14-3-3ζ, and the canonical 14-3-3-binding motif (Arg-Ser-Trp-Thr-Phe) contains two aliphatic hydroxy groups that are potential targets for kinases [38], we asked whether the phosphorylation of Ser⁵¹⁹ or Thr⁵²¹ in hIL-9Rα is essential. Competitive binding experiments were performed with non-phosphorylated, serine-phosphorylated or threonine-phosphorylated peptides, corresponding to residues 518–522 of hIL-9Rα (Figure 4, upper panel). Total lysates from 293 cells overexpressing hIL-9Rα were incubated with these peptides before incubation with lysates from 293 cells over-

Figure 4 Phosphorylated peptides corresponding to the 14-3-3-binding region (upper panel) within hIL-9Rα block the interaction between 14-3-3ζ and hIL-9Rα (lower panel)

Upper panel: peptide (P), phosphoserine-containing peptide (PS) and phosphothreonine-containing peptide (PT) sequences from the C-terminal residues 508–522 of hIL-9Rα. Lower panel: total lysates from 293 cells overexpressing hIL-9Rα were preincubated with control buffer (lanes 1 and 5), peptide (P) (lanes 2 and 6), phosphoserine-containing peptide (PS) (lanes 3 and 7) or phosphothreonine-containing peptide (PT) (lanes 4 and 8) (each at 1 mM), followed by the addition of lysates from 293 cells overexpressing 14-3-3ζ. The complexes were immunoprecipitated with anti-FLAG antibodies and Western blot analysis (WB) was performed with anti-FLAG (lanes 1–4) or anti-Myc (lanes 5–8) antibodies. Results are representative of three separate experiments. The positions of molecular mass markers are indicated at the right.

expressing 14-3-3ζ. Subsequent immunoprecipitation with anti-FLAG monoclonal antibody and immunoblot analysis with anti-FLAG antibody showed that non-phosphorylated or phosphorylated peptides did not affect the immunoprecipitation of hIL-9Rα (Figure 4, lower panel, lanes 1–4). However, the peptide phosphorylated at Ser⁵¹⁹ or Thr⁵²¹ inhibited the binding between hIL-9Rα and 14-3-3ζ (Figure 4, lower panel, lanes 7 and 8 respectively). These results suggest that the phosphorylation of Ser⁵¹⁹ or Thr⁵²¹ is essential for the binding of hIL-9Rα to 14-3-3ζ.

IL-9Rα binds to helix I in 14-3-3ζ

To identify the binding domain(s) in 14-3-3ζ responsible for its interaction with hIL-9Rα, we used various 14-3-3ζ constructs that have previously been characterized [35]. In brief, wild-type

Figure 5 Binding of hIL-9R α to 14-3-3 ζ truncation mutants

Upper panel: wild-type (wt) 14-3-3 ζ contains 246 amino acid residues and is composed of nine α -helices from A to I, starting from the N terminus. Each α -helix is represented by a box, and non-helical regions are shown as lines. Truncation mutants of 14-3-3 ζ were constructed and expressed as described [35]. Lower panel: 293 cells were transfected with 5 μ g of FLAG-epitope-tagged hIL-9R α (pFlagIL-9Rwt). Whole-cell lysates were incubated with Sepharose beads conjugated with equivalent amounts [35] of MBP as negative control, wild-type 14-3-3 ζ (WT) or truncation mutants 14-3-3 ζ (1–231), 14-3-3 ζ (1–209), 14-3-3 ζ (1–187), 14-3-3 ζ (1–162), 14-3-3 ζ (1–109), 14-3-3 ζ (1–31), 14-3-3 ζ (33–246), 14-3-3 ζ (136–246), 14-3-3 ζ (188–246), 14-3-3 ζ (136–209), 14-3-3 ζ (188–231), 14-3-3 ζ (188–209) and 14-3-3 ζ (202–231). After three washes, beads bound to hIL-9R α complexes were separated by SDS/PAGE and detected by Western blotting with anti-(hIL-9R α) antibody. The position of hIL-9R α is shown by an arrow. The amount of various mutant 14-3-3 ζ fusion proteins with MBP conjugated to Sepharose beads was estimated as described [35]. Results are representative of three separate experiments. The positions of molecular mass markers are indicated at the right.

and mutant proteins of 14-3-3 ζ were expressed as fusion proteins with MBP, purified and conjugated to Sepharose beads [35] (Figure 5, upper panel). FLAG-tagged hIL-9R α was overexpressed in 293 cells and whole cell lysates were incubated with MBP-conjugated beads or beads conjugated with wild-type or mutant 14-3-3 ζ . Western blot analysis with anti-(hIL-9R α) antibody showed that hIL-9R α bound to wild-type 14-3-3 ζ and mutants 14-3-3 ζ (1–231), 14-3-3 ζ (33–246), 14-3-3 ζ (136–246), 14-3-3 ζ (188–246) and 14-3-3 ζ (202–231) (Figure 5, lower panel). 14-3-3 ζ mutants lacking the helix I domain did not show binding to hIL-9R α , suggesting that helix I (residues 202–231) is crucial for binding to hIL-9R α . Interestingly, one mutant containing helices H and I of 14-3-3 ζ (188–231) did not bind to hIL-9R α . It is possible that helix H might bind to other molecules that prevent the interaction between 14-3-3 ζ and hIL-9R α . Although mutants 14-3-3 ζ (1–231), 14-3-3 ζ (33–246), 14-3-

3 ζ (136–246) and 14-3-3 ζ (188–246) contain helix H, the presence of other helices or residues 231–246 might have eliminated the effect of helix H, resulting in the complex formation between 14-3-3 ζ and hIL-9R α .

DISCUSSION

In the present study we have identified 14-3-3 ζ as one of the molecules that interact with the intracellular domain of hIL-9R α . To our knowledge this is the first demonstration of 14-3-3 interaction with a non-tyrosine kinase receptor. The motif in hIL-9R α that interacts with 14-3-3 ζ is different from motifs that are known to interact with 14-3-3 proteins in other systems. In addition, we showed that 14-3-3 ζ does not interact with IL-4R α , which, like IL-9R α , belongs to the IL-2R γ superfamily. The results suggest that 14-3-3, through its interaction with IRS-1

(results not shown; see below), might in part determine the specificity of IL-4 and IL-9. This is consistent with our previous observation that IL-4 and IL-9 stimulated the tyrosine phosphorylation of IRS-1 through different mechanisms (T. Yin, X.-Y. Wang and Y.-C. Yang, unpublished work).

The rationale for co-expressing LexA-IL-9R α and the kinase domain of JAK1 kinase on a bait plasmid in the two-hybrid system was based on the observations that JAK1 autophosphorylated tyrosine as well as serine/threonine residues and that the phosphorylation of hIL-9R α is required for signalling [9]. Our results showed that the interaction between 14-3-3 ζ and the intracellular domain of hIL-9R α in a yeast two-hybrid assay is only double that with the bait plasmid containing the kinase domain of JAK1. It is possible that the interaction between hIL-9R α and 14-3-3 ζ results from residues phosphorylated by endogenous yeast kinase activity and that the expression of the kinase domain of JAK1 further increased the interaction between hIL-9R α and 14-3-3 ζ .

We showed that 14-3-3 ζ interacts with the C-terminal five residues of the intracellular domain of hIL-9R α (Arg-Ser-Trp-Thr-Phe). In addition, a point mutation from Arg-Ser⁵¹⁹-Trp-Thr-Phe to Arg-Ala⁵¹⁹-Trp-Thr-Phe abolished the binding between hIL-9R α and 14-3-3 ζ . This observation is not so surprising because a specific phosphoserine-containing motif Arg-Ser-Xaa-pSer-Xaa-Pro for binding to 14-3-3 has been suggested and confirmed with phosphoserine-orientated peptide libraries [39,40]. Specific serine-phosphorylation sites mediating 14-3-3 interaction have also been identified for insulin-like growth factor ('IGF-I') receptor and p53 [27,41,42]. The Arg-Ser-Trp-Thr-Phe motif in the intracellular domain of hIL-9R α is different from the consensus 14-3-3 motif (Arg-Ser-Xaa-pSer-Xaa-Pro). We have further shown by phosphopeptide competition that Arg-pSer-Trp-Thr-Phe or Arg-Ser-Trp-pThr-Phe abolished the binding between hIL-9R α and 14-3-3 ζ , suggesting a new phosphoserine/phosphothreonine-containing motif for 14-3-3 binding. Although the biological function of the serine/threonine phosphorylation of hIL-9R α is not known, the binding of 14-3-3 ζ might protect phosphorylated sites of hIL-9R α from phosphatases, as has been proposed for Raf-1 [43,44]. Alternatively, a canonical 14-3-3-binding motif (Arg-Ser-Trp-Thr-Phe) with two aliphatic hydroxy groups might be a potential substrate for yet unidentified kinases [38].

In the present study we also determined that helix I, encompassing residues 202–231 in the C-terminal domain of 14-3-3 ζ , is a binding site for hIL-9R α . This region has recently been shown by Gu and Du [35] to be required for binding to GPIb α and GPIb-IX. In addition, Wang et al. [45] showed that the binding of c-Raf, Cbl and c-Bcr to 14-3-3 ζ requires helices G and I. The important role for helix I has also been demonstrated for Rad24, a 14-3-3 protein in fission yeast, in which mutations of a putative nuclear-export signal in Rad24 (the region of residues 219–234) impaired the nuclear exclusion of Rad24, the damage-included nuclear export of Cdc25 and the damage checkpoint [17]. This suggests that proteins that bind to helix I of 14-3-3 might regulate cell cycle progression by competing for binding with Cdc25 [46]. The physiological significance of binding between helix I of 14-3-3 ζ and IL-9R α requires further experimental studies.

Our results in Figure 5 (lower panel) suggested that the surrounding sequences of helix I might also contribute to the binding of hIL-9R α to 14-3-3 ζ . This is not surprising because it has been suggested that the specificity of interactions between 14-3-3 and its target protein does not result from a different specificity for the phosphopeptide-binding motifs but rather arises from contacts made on the variable surface of 14-3-3

outside the binding cleft [40]. The highly conserved motif for binding to 14-3-3 proteins is probably not sufficient to determine an exact specificity for the binding of different 14-3-3 isoforms; additional 14-3-3 isotype-specific structures might be required. Furthermore, a variety of intracellular signalling proteins have specific interactions with particular 14-3-3 isoforms [24,27,29,30]. The interactions between 14-3-3 and other molecules therefore is determined by the specific 14-3-3-binding motifs, their phosphorylation status in a particular 14-3-3 ligand and specific helical domains in the 14-3-3 structure.

We have shown here that 14-3-3 ζ binds to hIL-9R α but not to mL-4R α . Although IL-9 and IL-4 share some common signalling pathways [10] and the intracellular domain of IL-4R α contains Arg-Ser-Xaa-Xaa-Xaa and Arg-Ser-Xaa-Ser-Xaa-Xaa sequences, none of them match exactly the motif in IL-9R α or the suggested 14-3-3-binding motifs (Arg-Ser-Xaa-Ser-Xaa-Pro). We have shown that 14-3-3 ζ also binds to IRS-1 (results not shown) and that IRS-1 is tyrosine-phosphorylated after stimulation by IL-4 and IL-9 [10]. Furthermore, the deletion of the pleckstrin homology ('PH') domain in IRS-1 abolished the tyrosine phosphorylation of IRS-1 induced by mL-9 but did not affect tyrosine phosphorylation induced by mL-4 (T. Yin, X.-Y. Wang and Y.-C. Yang, unpublished work), indicating that different mechanisms are involved in IRS-1 tyrosine phosphorylation mediated by IL-4 and IL-9. 14-3-3 and IRS proteins therefore seem to be potential candidates for determining cytokine specificity. The identification of serine/threonine kinases responsible for the phosphorylation of hIL-9R α and its subsequent binding to 14-3-3 will be essential in unravelling the mechanisms involved.

In summary, we have found that: (1) 14-3-3 ζ associates with the intracellular hIL-9R α , (2) a new binding motif in the C-terminal domain of hIL-9R α is required for binding to 14-3-3 ζ , (3) interaction between hIL-9R α and 14-3-3 ζ requires the phosphorylation of Ser⁵¹⁹ or Thr⁵²¹, and (4) helix I of 14-3-3 ζ is important for its interaction with hIL-9R α . Further studies will be necessary to elucidate the physiological role of the association of 14-3-3 ζ with hIL-9R α in the IL-9 signalling pathway.

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