Recombinant Human Interleukin-9 Induces Protein Tyrosine Phosphorylation and Synergizes With Steel Factor To Stimulate Proliferation of the Human Factor-Dependent Cell Line, M07e

By Keisuke Miyazawa, Paul C. Hendrie, Young-June Kim, Charlie Mantel, Yu-Chung Yang, Byoung Se Kwon, and Hal E. Broxmeyer

Human interleukin-9 (IL-9) was originally identified and cloned based on its stimulatory effect on proliferation of human myeloid cell line, M07e. IL-9 synergized with Steel factor, the ligand for the c-kit product, to stimulate M07e cell proliferation. To investigate potential mechanisms for this, IL-9 was assessed for effects on protein tyrosine kinase activities in M07e cells by immunoblotting with anti-phosphotyrosine monoclonal antibody; results were compared with those of Steel factor alone and in combination with IL-9, and those of 12-0-tetradecanoyl phorbol-13-acetate (TPA). Recombinant human IL-9 (10 ng/mL) rapidly and transiently induced or enhanced at least four tyrosine phosphorylated protein bands with molecular weights of 105, 97, 85, and 81 Kd. This tyrosine phosphorylation pattern was different from that generated by recombinant murine Steel factor or TPA stimulation and the combination of IL-9 and Steel factor did not change the IL-9-induced pattern. IL-9–induced tyrosine-phosphorylated bands were completely blocked by treatment of IL-9 with anti–IL-9 antibody under conditions that also neutralized the synergistic effect of IL-9 with Steel factor on M07e cell proliferation. Genistein, a tyrosine kinase inhibitor, blocked phosphorylation of IL-9 and Steel factor-induced bands. Unlike Steel factor or TPA, IL-9 did not appear to stimulate phosphorylation of 42-Kd mitogen-activated protein (MAP) kinase or Raf-1, or enhance MAP kinase activity. MAP kinase and Raf-1 are serine/threonine kinases that are phosphorylated and activated by many growth factors and by agonists for protein kinase C. While the combination of IL-9 plus SLF did not appear to induce phosphorylation of new bands not already seen with either IL-9 or SLF alone, or enhance the phosphorylation of those bands seen with either cytokine alone, the results suggest that IL-9 activates specific and unique signal transduction pathways.

MATERIALS AND METHODS

Cytokines and reagents. Highly purified recombinant human (rhu) IL-9 (1.2 × 10^6 U/mg) and anti–IL-9 antibody were obtained from Genetics Institute (Cambridge, MA). Highly purified recombinant murine Steel factor (rmuSLF) and rhuGM-CSF (4 × 10^7 U/mg) were a kind gift from Dr Douglas E. Williams (Immunex Corp, Seattle, WA).23 Affinity-purified rabbit polyclonal anti-Raf-1 antibody and a synthetic peptide corresponding to amino acid residues 315 to 326 of v-raf were a generous gift from Kenneth Wood (Dana Farber, Boston, MA).24 Mouse anti-phosphotyrosine monoclonal antibody (MoAb) (4G10) and genistein were obtained from Upstate Biotechnology Inc (Lake Placid, NY). Mouse anti-MAP kinase MoAb, alkaline phosphatase conjugated goat anti-mouse IgG (H + L), and goat antirabbit IgG (H + L) were purchased from Zymed Laboratories, Inc (San Francisco, CA). 12-0-tetradecanoyl phorbol-13-acetate (TPA) and calf intestine alkaline phosphatase were purchased from Sigma Chemical Co (St Louis, MO).

M07e cell line. The human factor-dependent cell line M07e was obtained from Aggie Ciarletta (Genetics Institute). The biologic specific saturable membrane surface IL-9 binding sites have been demonstrated by others in murine factor-dependent T-cell lines, mast cell lines, and macrophages.13 However, little is known about the biochemical properties of the IL-9 receptor and its post-ligand receptor-binding signaling system. In this report, we demonstrate that human IL-9 specifically induced rapid and transient protein tyrosine phosphorylation in M07e cells, which closely correlated with the expression of the synergistic effect of IL-9 with SLF on the proliferation of this cell line. In addition, IL-9 did not stimulate phosphorylation of Raf-1 or phosphorylation or kinase activity of 42-Kd mitogen-activated protein (MAP) kinase, two serine/threonine-specific kinases that are phosphorylated by various growth factors including GM-CSF and SLF as well as protein kinase C activation.14-22 These data suggest specific and unique signaling pathways mediated by human IL-9.
characteristics of this cell line have been previously described.\textsuperscript{2,12} The cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 100 U/mL rhuGM-CSF. [\textsuperscript{3}H]Thymidine incorporation was used to measure cell proliferation by incubation in quadruplicate aliquots of M07e cells cultured in flat-bottom microtiter plates (150 \( \mu \)L/well) with or without various concentrations of rhuIL-9 and/or rmuSLF for 72 hours at 37°C. Cells were pulse labeled with [\textsuperscript{3}H]thymidine (Amersham, Arlington Heights, IL) at 0.5 \( \mu \)Ci/well for a final 4 hours. For the phosphorylation experiments, exponentially growing M07e cells were washed and incubated for 18 hours at 37°C in serum-free RPMI-1640 medium containing 0.5% bovine serum albumin (BSA; Sigma). After "factor-starvation," cells were washed once with serum-free medium and then stimulated with rhuIL-9, rmuSLF, rhuIL-9 plus rmuSLF, or TPA for various periods of time at 37°C.

After factor stimulation, M07e cells were immediately washed with ice-cold phosphate-buffered saline (PBS) containing 100 mmol/L sodium fluoride (Sigma) and 2 mmol/L sodium orthovanadate (Sigma) and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40 (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 0.15 U/mL aprotinin, 10 mmol/L EDTA, 10 \( \mu \)g/mL leupeptin, 100 mmol/L sodium fluoride [NaF], 2 mmol/L sodium orthovanadate) at 4°C for 30 minutes. Insoluble material was removed by centrifugation at 4°C for 15 minutes at 10,000g. Total protein content of the cell lysates were determined by Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Cell lysates were used for immunoprecipitation and immunoblotting.

**Immunoblotting and immunoprecipitation.** Immunoblotting was performed as previously described.\textsuperscript{16} Briefly, the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were electrophoretically transferred onto Immobilon-P membrane (Millipore, Bedford, MA). After blocking residual binding sites on the transfer membrane by TBST then incubated with alkaline phosphatase-conjugated goat antimouse IgG (H + L) MoAb (diluted 1:1,000 in TBST) for 1 hour at room temperature. Antibody reactions were developed for 10 to 30 minutes in a solution containing 100 mmol/L Tris-HCl pH 9.5, 150 mmol/L NaCl, 5 mmol/L MgCl\(_2\), 330 \( \mu \)g/mL nitro blue tetrazodium (NBT; Bio-Rad), 150 \( \mu \)g/mL 5-bromo-4-chloro-3-indolyolphosphate (BCIP; Bio-Rad). Enzymatic color development was stopped by rinsing the blots in deionized water.

Immunoprecipitations were performed by incubating cell lysates with the appropriate antibody for 3 to 6 hours at 4°C. Protein A-sepharose beads (Pierce, Rockford, IL) were used to collect the antigen-antibody complexes. The immunoprecipitates were washed five times with ice-cold TTBS (100 mmol/L Tris-HCl, pH 8.0, 0.5% Tween-20, 150 mmol/L NaCl) containing 2 mmol/L sodium orthovanadate and 100 mmol/L sodium fluoride, and three times with lysis buffer before analysis.

**Metabolic labeling of M07e cells.** For metabolic labeling with [\textsuperscript{3}P]orthophosphate, growth factor-starved cells were washed and resuspended at 10\(^5\) cells/mL in phosphate-free RPMI-1640 medium containing 0.5% BSA and incubated for 2 hours. Then cells were resuspended at 10\(^6\) cells/mL in phosphate-free medium and equilibrated with carrier-free [\textsuperscript{3}P]orthophosphate (Amersham) at 1 \( \mu \)Ci/mL for 90 minutes. Radiolabeled cells were stimulated with growth factors for various times and immediately lysed in lysis buffer as described above. After removing insoluble material by centrifugation, cell lysates were normalized for protein content and used for immunoprecipitation.

**MAP kinase activity.** An MAP kinase assay as reported by others\textsuperscript{26} was performed with some modifications. After growth factor stimulation as above, 3 \( \times 10^5\) cells were washed with ice-cold PBS and lysed in 200 \( \mu \)L cold lysis buffer (1% Triton X-100 [Sigma]), 0.5% NP-40, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L NaF, 0.2 mmol/L sodium orthovanadate, 0.2 mmol/L PMSF) for 15 minutes on ice. Insoluble material was removed by centrifugation at 4°C for 15 minutes at 10,000g. For assay, either whole lysates or anti-MAP kinase immunoprecipitates were used.

For immunoprecipitation, 50 \( \mu \)L lystate supernatant was incubated with 4 \( \mu \)g of mouse anti-MAP kinase (ERK1 and 2) MoAb (IgG\(_1\); Zymed) for 1 hour at 4°C. Subsequently, 5 \( \mu \)g of affinity-purified sheep anti-mouse IgG (Cappel Inc, Durham, NC) was added and incubated for 30 minutes at 4°C. MAP kinase was recovered using 50 \( \mu \)L protein G-Sepharose 4B conjugate suspension (Zymed) and washed.

For kinase assay, either 1 \( \mu \)L of whole lystate was added to 10 \( \mu \)L of reaction buffer (50 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl\(_2\), 50 mmol/L sodium orthovanadate, 5 \( \mu \)g/mL leupeptin) or immunoprecipitates were resuspended in 25 \( \mu \)L of reaction buffer. To the reaction mixture, 10 \( \mu \)Ci [\( \gamma \)-\textsuperscript{32}P]ATP (Amersham) and 0.5 mg/mL bovine myelin basic protein (MBP; Sigma) as substrate were added and these were incubated for 30 minutes at 30°C. To terminate the reaction, 1/5 vol of 5X concentrated SDS-PAGE sample buffer was added and boiled for 5 minutes. After centrifugation for 5 minutes, 10 \( \mu \)L of supernatant was analyzed by 12.5% SDS-PAGE, transferred onto Immobilon-P membrane, and visualized by autoradiography. The autoradiographic MBP bands were quantitated by scanning densitometry. After autoradiography, the membrane was stained with Coomassie blue and MBP bands were cut out and counts per minute (cpm) determined with a Beckman 6000IC scintillation counter (Beckman, Fullerton, CA).

**Densitometry and molecular weight analysis.** Scanning densitometry was performed using the Bio-Rad Video Densitometer model 620. The molecular weights presented in this report were derived by extrapolation of RI values of standard proteins using Bio-Rad gel analysis software employing quadratic methods to relate migration distance to molecular size in SDS-PAGE gels.

**Statistics.** All statistical tests were based on the assumption of an underlying Poisson distribution. The evaluation of the greater than additive effects between cytokines was obtained as \( \chi^2 \) tests arising from a regression procedure assuming a Poisson distribution.\textsuperscript{26}

**RESULTS**

**rhuIL-9 synergizes with SLF to stimulate M07e cell proliferation.** M07e cells were cultured for 72 hours in media containing 20% fetal calf serum (FCS) with various concentrations of rhuIL-9 or rmuSLF. [\textsuperscript{3}H]Thymidine incorporation was used as a measure of DNA synthetic rate. IL-9 slightly but significantly stimulated M07e cell proliferation at a concentration greater than 0.1 mg/mL, with maximum response observed at 100 ng/mL (Fig 1A). ED\(_{50}\) (effective dose) of rhuIL-9 was approximately 1 mg/mL in our system. rmuSLF alone began to stimulate at 5 ng/mL in a dose-dependent manner with plateau effects noted with \( \geq 5 \) mg/mL (Fig 1A). In the presence of SLF, IL-9 strikingly
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Enhanced M07e cell proliferation (Fig 1B). For example, simultaneous stimulation of 10 ng/mL IL-9 and 50 ng/mL SLF resulted in about an eightfold increase in proliferation when compared with that of 50 ng/mL SLF alone. These data extend our previous study demonstrating that IL-9 has a potent stimulating activity on M07e cell proliferation when combined with SLF, even though alone it shows only subtle effects.12

IL-9 stimulates protein tyrosine phosphorylation in a time-and a dose-dependent manner. Because protein-tyrosine kinase activation has been implicated in events leading to cell proliferation by many growth factors and mitogenic stimuli,27 we investigated IL-9--induced protein tyrosine phosphorylation in M07e cells (Fig 2). Cells were cultured under factor-free conditions for 18 hours and stimulated with 10 ng/mL IL-9 for various lengths of time at 37°C (Fig 2A). In over 30 separate experiments, IL-9 stimulation resulted in the consistent appearance or enhancement of four tyrosine phosphorylated bands with molecular weights of 105, 97, 85, and 81 Kd as measured by 7.5% SDS-PAGE analysis followed by immunoblotting with anti-phosphotyrosine MoAb. Other bands, including those seen at 140 and

![Image of Fig 1](https://example.com/image1)

**Fig 1.** Influence of IL-9, SLF, and IL-9 + SLF on proliferation of M07e cells. Cells, 10^5, were plated per well and each point was repeated in quadruplicate. Cells were exposed to cytokines for 72 hours and then pulsed with [3H]thymidine (0.5 μCi/well) for 4 hours. (A) Dose-response effect of rhuIL-9 or rmuSLF alone (*P < .001 vs control medium). (B) Dose-response to rhuIL-9 in the presence of rmuSLF (*P < .0001 for greater than additive effects).

![Image of Fig 2](https://example.com/image2)

**Fig 2.** Time and dose effects of IL-9--induced tyrosine phosphorylation in M07e cells. (A) M07e cells (10^5/mL) were stimulated with rhuIL-9 (10 ng/mL) for the indicated times. (B) Cells were cultured at the indicated concentrations of rhuIL-9 for 10 minutes. Cell lysates were analyzed by 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine MoAb according to Materials and Methods. The specificity of anti-phosphotyrosine MoAb was confirmed by incubating the blots with this antibody in the presence of excess phosphotyrosine, phosphoserine, or phosphothreonine. All immunoreactive bands shown here were completely eliminated in the presence of 1 mmol/L phosphotyrosine, while no effect was seen with phosphoserine and phosphothreonine.
Table 1. Effect of Anti-IL-9 Antibody on M07e Cell Proliferation Induced by IL-9 and/or SLF

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Pre-immune Serum (1:100)</th>
<th>α-IL-9 Antibody (1:100)</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td>IL-9 (1 ng/mL)</td>
<td>0.12 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>IL-9 (10 ng/mL)</td>
<td>0.56 ± 0.02</td>
<td>0.22 ± 0.01*</td>
</tr>
<tr>
<td>SLF (50 ng/mL)</td>
<td>6.0 ± 0.4</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>IL-9 (1 ng/mL) + SLF</td>
<td>19.7 ± 1.5</td>
<td>7.7 ± 1.3t</td>
</tr>
<tr>
<td>IL-9 (10 ng/mL) + SLF</td>
<td>25.3 ± 0.1</td>
<td>9.3 ± 0.3t</td>
</tr>
</tbody>
</table>

10^4 M07e cells were plated per well and cultured with IL-9 and/or SLF at the indicated concentrations in the presence of anti-IL-9 antibody (1:100) or pre-immune serum, for 72 hours at 37°C. Then cells were pulse labeled with [3H]thymidine (0.5 μCi/well) for 4 hours. All numbers shown are the mean ± SD.

*P < .01 v pre-immune serum.

†P < .001 v pre-immune serum.

60 Kd in Fig 2A, were not reproducibly induced/enhanced. Phosphorylated bands with molecular weights lower than 60 Kd were not induced/enhanced even by 10% SDS-PAGE analysis (data not shown). These tyrosine phosphorylated bands were rapidly induced within 2 minutes after IL-9 stimulation and persisted for 45 minutes. After 90 minutes the tyrosine phosphorylation pattern returned to that of unstimulated cells. When cells were treated for 10 minutes with 0.001 to 100 ng/mL of IL-9, a similar set of tyrosine phosphorylation bands were induced at 0.1 ng/mL of IL-9, and their density increased in a dose-dependent manner (Fig 2B).

The p81 and p85 bands behaved in an opposite fashion with respect to their density. As shown in the time sequence study presented in Fig 2A, after 5 minutes of exposure to IL-9, the density of the lower band was greater than that of the upper band. However, after 30 minutes the lower band had become weaker and the upper band had become denser. Finally, at 45 minutes after IL-9 stimulation the p81 band had disappeared and only the p85 band was detectable. Since other phosphoproteins implicated in mitogenic signal transduction, such as Raf-1 and p561ck, have been shown to have very similar retard migration patterns on SDS-PAGE gels by hyperphosphorylation, the p81/p85 bands might possibly represent a single protein with different levels of phosphorylation.

Anti-IL-9 antibody blocks the synergistic effect of IL-9 and SLF on M07e cell proliferation and blocks IL-9-induced tyrosine phosphorylation. To substantiate the specificity of IL-9 we examined the effect of anti-IL-9 antibody on both tyrosine phosphorylation induction and the stimulatory effect on M07e cell proliferation induced by IL-9. As shown in Table 1, anti-IL-9 antibody (1:100) significantly reduced entirely or almost entirely the synergistic effect of IL-9 plus SLF on M07e cell proliferation as evaluated by [3H]thymidine incorporation.

Protein tyrosine phosphorylation was investigated under the same conditions (Fig 3). IL-9–induced tyrosine phosphorylated bands were completely blocked in the presence of anti-IL-9 antibody, whereas this antibody had no effect on SLF–induced tyrosine phosphorylated bands. In addition, pretreatment of the cells with genistein, a tyrosine kinase inhibitor, blocked tyrosine phosphorylation induction by both IL-9 and SLF.

Comparison of tyrosine phosphorylation patterns induced by IL-9, SLF, and TPA. We previously reported that SLF stimulation induced c-kit receptor tyrosine kinase activation and resulted in the induction of at least 12 tyrosine phosphorylated bands in M07e cells. The major bands had molecular weights of 145, 120, 110, 98, 62, 55, and 42 Kd.

As shown in Fig 4, the protein tyrosine phosphorylation pattern induced by IL-9 stimulation was different from those generated by SLF or TPA (an agonist for protein kinase C). Also, we could not detect any new bands or further enhancement of the bands induced by either IL-9 or SLF, when M07e cells were stimulated by the combination of IL-9 plus SLF (data not shown). The 97-Kd band induced by IL-9 stimulation migrated close to one of the bands induced by SLF (Fig 4, lanes 2 and 3). However, multiple experiments showed that the 97-Kd band always migrated at a slightly lower position and appeared as a broader band than that induced by SLF. As reported by

Fig 3. Effect of anti-IL-9 antibody and genistein on protein tyrosine phosphorylation induced by IL-9 or SLF. (A) Anti-IL-9 antibody or pre-immune serum (1:100) were pre-incubated with control medium, or medium plus rhuIL-9 (10 ng/mL), or rmuSLF (50 ng/mL) for 30 minutes at 37°C. Then M07e cells were suspended at 1 x 10^7/mL with the above combinations for 10 minutes at 37°C. (B) M07e cells (1 x 10^6/mL) were incubated with 40 μg/mL genistein for 1 hour at 37°C before factor stimulation. The cell density was adjusted to 1 x 10^7/mL and cells were treated with control medium or medium plus rhuIL-9 (10 ng/mL) or SLF (50 ng/mL) for 10 minutes at 37°C. Cell lysates were analyzed by 7.5% SDS-PAGE and immunoblotted with antiphosphotyrosine MoAb.
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Fig 4. Comparative analysis of stimulated tyrosine phosphorylation patterns with IL-9, SLF, and TPA. After treatment with control medium, or medium plus 10 ng/mL rhIL-9, 50 ng/mL rmuSLF, or 160 nmol/L TPA for 10 minutes, M07e cells were lysed and analyzed by 7.5% SDS-PAGE and immunoblotted with anti-phosphotyrosine MoAb. Others, the 98-kd tyrosine phosphorylated protein induced by SLF stimulation appears to be identical with the proto-oncogene product Vav that contains leucine- and cysteine-rich regions as well as an SH2 domain. It is also noteworthy that a 42-kd tyrosine phosphorylated band was induced by SLF and TPA but not by IL-9 stimulation (Fig 4, lanes 3 and 4, the lower arrowheads).

IL-9 does not induce phosphorylation of either MAP kinase or Raf-1 or enhance MAP kinase activity. MAP kinase and Raf-1, both serine/threonine-specific protein kinases, are ubiquitously expressed in various tissues and have been shown to be phosphorylated and their kinase activities activated by many growth factors and mitogenic stimuli, implicating their importance as components of mitogenic signals. Therefore, our investigation focused on these two molecules and whether or not IL-9 stimulation induced their phosphorylation in M07e cells.

As shown in Fig 5A, immunoblotting of the whole cell lysates with anti-MAP kinase MoAb after factor stimulation showed that the detected amount of protein was the same under all conditions. To examine if IL-9 induced MAP kinase tyrosine phosphorylation, anti-MAP kinase immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibody (Fig 5B). A 42-kd tyrosine phosphorylated band was detected in immunoprecipitation by either SLF or TPA stimulation but not by IL-9 stimulation.

We also evaluated effects on MAP kinase activity using myelin basic protein as a substrate (Table 2). While MAP kinase activity of lysates and immunoprecipitates was enhanced by SLF and TPA, IL-9 did not enhance MAP kinase activity, and the combination of IL-9 plus SLF did not have any greater effect than that seen with SLF alone.

Next, whole cell lysates were immunoblotted with anti-Raf-1 antibody (Fig 6A). Retarded migration of Raf-1 on 7.5% SDS-PAGE was detectable after SLF and TPA, but not after IL-9, stimulation. This retarded migration of Raf-1 has been reported by others to represent the hyperphosphorylated state of Raf-1 protein. Also, 32P-labeled proteins were immunoprecipitated with anti–Raf-1 antibody. As shown in Fig 6B, IL-9 stimulation did not enhance Raf-1 phosphorylation, whereas SLF and TPA stimulation increased phosphorylation of Raf-1 threefold to fourfold as detected by densitometric analysis. Not shown is that the combination of IL-9 and SLF did not further enhance Raf-1 phosphorylation over that noted by treatment of cells with SLF alone.

DISCUSSION

Many growth factor receptors stimulate protein tyrosine phosphorylation after ligand binding. This event is thought to be an essential part of the signal transduction mechanism that mediates the cellular response.
for lysates and immunoprecipitates. Control cpm for lysates and immunoprecipitate. Control values on which the percent of controls for this were based were 3.85 and 8.70, respectively, for lysates and immunoprecipitates. Control cpm for lysates and immunoprecipitates were 7,750 and 1,13, respectively. Data shown for lysates and immunoprecipitates were evaluated.

While the IL-9 receptor has not yet been purified and little is known about the biochemical properties of this receptor, several lines of evidence support the idea that these phosphoproteins are specifically induced by IL-9 stimulation and may be important components of post-ligand receptor binding signaling pathways of IL-9. First, similar concentrations of IL-9 were involved in the induction of protein tyrosine phosphorylation and stimulation of M07e cell proliferation. Second, the IL-9-induced protein tyrosine phosphorylation pattern was different from those generated by SLF or TPA stimulation. Third, phosphorylation was blocked by anti-IL-9 antibody under the same conditions that neutralized the synergistic effect of IL-9 plus SLF on M07e cell proliferation.

So far, the relationship between any of the phosphoproteins detected in our studies with known tyrosine phosphorylated substrates is not known. Specific membrane surface binding sites for IL-9 have been studied in murine T-cell lines, mast cell lines, and macrophages. Based on these data as well as cross-linking studies, the IL-9 receptor appears to consist of a 64-Kd glycoprotein, the molecular weight of which is reduced to 54 Kd by treatment with N-glycosidase F. Because the molecular weight of the tyrosine phosphorylated bands detected in our experiments do not correspond with those of the murine IL-9 receptor, these phosphoproteins may not be derived from IL-9 receptor autophosphorylation but may represent the substrates of activated protein tyrosine kinase(s) that may play a role in signal transduction by IL-9. Identification and purification of these phosphoproteins as well as the IL-2,40 IL-3,17-19 GM-CSF,17-19 T-cell antigen,41 to be inappropriate for use in this context.

The following concentrations were used: 10 ng/mL IL-9, 50 ng/mL SLF, 160 nmol/L TPA. Peak area refers to the area of the densitometrically scanned autoradiographic band. Control values on which the percent of controls for this were based were 3.85 and 8.70, respectively, for lysates and immunoprecipitates. Control cpm for lysates and immunoprecipitates were 7,750 and 1,13, respectively. Data shown for lysates and immunoprecipitates were from separate experiments, although the results are representative of two experiments in which both lysates and immunoprecipitates were evaluated.

In this report, we demonstrated that human IL-9 stimulation induced at least four tyrosine phosphorylated bands. While the IL-9 receptor has not yet been purified and little is known about the biochemical properties of this receptor, several lines of evidence support the idea that these phosphoproteins are specifically induced by IL-9 stimulation and may be important components of post-ligand receptor binding signaling pathways of IL-9. First, similar concentrations of IL-9 were involved in the induction of protein tyrosine phosphorylation and stimulation of M07e cell proliferation. Second, the IL-9-induced protein tyrosine phosphorylation pattern was different from those generated by SLF or TPA stimulation. Third, phosphorylation was blocked by anti-IL-9 antibody under the same conditions that neutralized the synergistic effect of IL-9 plus SLF on M07e cell proliferation.

Table 2. Effects of IL-9 and SLF, Alone and in Combination, and TPA on MAP Kinase Activity in M07e Cells

<table>
<thead>
<tr>
<th>Agents</th>
<th>Lysates</th>
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<th>Immunoprecipitates</th>
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<tr>
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<td>Peak Area</td>
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<td>IL-9</td>
<td>109</td>
<td>94</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>SLF</td>
<td>162</td>
<td>133</td>
<td>150</td>
<td>140</td>
</tr>
<tr>
<td>IL-9 + SLF</td>
<td>174</td>
<td>170</td>
<td>143</td>
<td>154</td>
</tr>
<tr>
<td>TPA</td>
<td>257</td>
<td>364</td>
<td>145</td>
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</table>

The following concentrations were used: 10 ng/mL IL-9, 50 ng/mL SLF, 160 nmol/L TPA. Peak area refers to the area of the densitometrically scanned autoradiographic band. Control values on which the percent of controls for this were based were 3.85 and 8.70, respectively, for lysates and immunoprecipitates. Control cpm for lysates and immunoprecipitates were 7,750 and 1,13, respectively. Data shown for lysates and immunoprecipitates were from separate experiments, although the results are representative of two experiments in which both lysates and immunoprecipitates were evaluated.

Interestingly, IL-9 did not induce/enhance phosphorylation of MAP kinase (Fig 5) or Raf-1 (Fig 6), or activity of MAP kinase (Table 2). We did not evaluate Raf-1 kinase activity because presently the specific substrate for Raf-1 kinase activity has not been identified and H1 histone that has been used previously as a substrate for Raf-1 kinase activity by us and others has recently been demonstrated

![Fig 6. Raf-1 phosphorylation state after treatment with IL-9, SLF, or TPA. (A) M07e cells were treated with control medium, medium plus 10 ng/mL huIL-9, 50 ng/mL rmuSLF, or 160 nmol/L TPA for 10 minutes at 37°C. Whole cell lysates were separated by 7.5% SDS-PAGE and immunoblotted with anti-Raf-1 antibody. (B) M07e cells were labeled with carrier-free [32P] orthophosphate in vivo as described in Materials and Methods. After treatment with the factors as described above, the cell lysates were immunoprecipitated with anti–Raf-1 antibody. Immunoprecipitates were separated by 7.5% SDS-PAGE and Raf-1 was visualized by autoradiography. Raf-1 bands were completely blocked by the addition to the cell lysates of Raf-1 synthetic peptide before immunoprecipitation, demonstrating the specificity of the anti–Raf-1 antibody (data not shown).](image-url)
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and erythropoietin receptors, which lack intrinsic tyrosine kinase activity yet provoke the tyrosine phosphorylation of cellular proteins.

In M07e cells, we and others have previously reported that both SLF and GM-CSF stimulate Raf-1 phosphorylation and enhance Raf-1-associated kinase activity, suggesting Raf-1 as one of the shared signaling pathways of both factors in this cell line. However, combined stimulation of SLF and GM-CSF did not further enhance Raf-1 phosphorylation and Raf-1-associated kinase activation over that noted by SLF alone, whereas the combination of SLF plus GM-CSF strongly synergized to stimulate M07e proliferation. Even though simultaneous stimulation with IL-9 and SLF resulted in a striking increase in M07e cell proliferation (Fig 1), no further enhanced phosphorylation of Raf-1 was observed when compared with that of SLF alone (data not shown). This implies that there might be other mitogenic signaling pathways for these factors that do not involve Raf-1 kinase.

Another serine/threonine kinase, MAP kinase, also has been reported to be phosphorylated in response to many growth factors including insulin, nerve growth factor (NGF), GM-CSF, and SLF as well as agonists for protein kinase C. Because MAP kinase activation requires phosphorylation on both tyrosine and threonine residues in this molecule, MAP kinase may function at a point of convergence between different signaling pathways. However, recent reports demonstrated the existence of intramolecular autophosphorylation of MAP kinase on tyrosine and threonine residues in vitro. The in vivo functions of MAP kinase remain to be firmly established, but evidence suggests that it participates in a kinase cascade resulting in activation of an S6 kinase and phosphorylation of ribosomal protein S6. Furthermore, a very recent report demonstrated that MAP kinase phosphorylated c-jun, a component of the AP-1 transcription factor family in vitro. Phosphorylation of c-jun specifically occurred on positive regulatory sites for transacting activity of c-jun, implying that MAP kinase directly participates in mitogenic signaling of many growth factors. The data presented in this report indicate that IL-9 does not induce/ enhance phosphorylation of MAP kinase (42 Kd) or Raf-1 and does not affect MAP kinase activity. Also, the combination of IL-9 plus SLF does not appear to affect these proteins to any greater extent than that seen in the presence of SLF alone. Therefore, IL-9 may involve unique signaling pathways.

ACKNOWLEDGMENT

We thank Drs Douglas E. Williams of Immunex Corporation for providing rmu Steel factor, Katherine Turner and Ed Alderman of Genetics Institute for rhuIL-9 and anti–IL-9 antibody, and Kenneth Wood of Dana Farber Cancer Institute for the gift of anti–Raf-1 antibody and for his and Dr Thomas Robert’s personal comments to us concerning the unsuitability of using H1 histone as a substrate for measuring Raf-1 kinase activity. We also thank Becki Robling and Linda Cheung for preparation of the manuscript.

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