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Fungal Immunomodulatory Protein from *Flammulina velutipes* Induces Interferon-γ Production through p38 Mitogen-Activated Protein Kinase Signaling Pathway

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FIP-fve is a fungal immunomodulatory protein purified from *Flammulina velutipes*, an edible golden needle mushroom thought to possess potent immunomodulatory properties. When examined for its effects on lymphocytes, FIP-fve exhibited potent mitogenic effects on human peripheral blood lymphocytes, inducing G1/G0 to S phase proliferation. T cells activated by FIP-fve show significant production and secretion of interferon-γ (IFN-γ) associated with intercellular adhesion molecule 1 expression but low detectable levels of interleukin-4 in vitro or in vivo. However, SB203580, the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor, can fully abolish the production of IFN-γ induced by FIP-fve. At the same time, SB203580 only partially prevents the lymphocytes from progressing from G1 to S phase of the cell cycle. These findings demonstrate that FIP-fve is a potent T-cell activator, mediating its effects via cytokine regulation of p38 MAPK. The immunoprophylatic effects of FIP-fve in Th2-mediated allergic anaphylaxis are believed to be associated with the ability of FIP-fve to enhance activation of IFN-γ-releasing Th1 cells.

KEYWORDS: Fungal immunomodulatory protein; IFN-γ; p38

INTRODUCTION

A fungal immunomodulatory protein (FIP), FIP-fve, was isolated from *Flammulina velutipes*, which is a popular edible mushroom in the Orient and is named golden needle mushroom. FIP-fve consists of 114 amino acid residues and bears primary and secondary structural similarities to the human immunoglobulin (Ig) heavy chain (*1*, *2*). FIP-fve exhibits the potential to modulate immune responses similar to LZ-8 isolated from the Oriental medicinal fungus *Ganoderma lucidium*. Moreover, FIP-fve and LZ-8 have a high degree of amino acid sequence homology, with 70 (61.4%) invariant amino acid residues (*1*). FIP-fve is mitogenic in vitro for human peripheral blood lymphocytes and induces a bell-shaped dose−response curve similar to that for lectin mitogens. We had previously demonstrated that the edema reaction and systemic anaphylaxis reaction can be prevented by repeat administration of FIP-fve, in vivo (*1*). Thus FIP-fve seems to be a new mitogen and immunomodulator with therapeutic potential, whose mechanisms of action need to be clarified. To better assess the immunomodulatory potential of FIP-fve, we sought to determine the effect of FIP-fve on activation of peripheral blood mononuclear cells (PBMCs).

Helper T cells are heterogeneous with regard to cytokine secretion and their functions. It is well-known that Th1 cells produce mainly interleukin-2 (IL-2) and interferon-γ (IFN-γ), while allergy-associated Th2 cells predominantly produce interleukins-4 and -6 (IL-4 and IL-6) (*3*, *4*). The production of IFN-γ by Th1 cells can amplify Th1 development and inhibit the proliferation of Th2 cells, whereas IL-4 produced by Th2 cells blocks the inflammatory function of Th1 cells (*5*). The magnitude and duration of the production of the initial allergic trigger, IgE, are mainly determined by the cooperative effect between antagonistic IL-4 and IFN-γ functions (*5*, *6*). Therefore, modulation of the functional differentiation of allergen-specific Th cells may have clinical utility in the treatment of allergic diseases.

Mitogen-activated protein kinase (MAPK) pathways play a critical role in the activation of T cells during the immune response (*7*). Several studies have shown the involvement of the ERK pathway. The positive selection of T cells in the thymus and in T-cell activation (*8*). It has also been shown that JNK is activated during T-cell activation, where this molecule appears to play a role in integrating signals initiated at the T-cell receptor

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(TcR) complex (*9*). p38 is involved in positive selection in early thymocyte development (*10*). In addition, p38 MAPK plays a critical role in the differentiation of thymocytes from CD4(+)-CD8(+) double-positive (DP) precursors to mature CD4(+) and CD8(+) single-positive (SP) thymocytes during late intrathymic development (*11*). Since p38 MAP kinase can be activated by different proinflammatory cytokines and has been implicated in the expression of specific cytokines, it is possible that this kinase plays a role in the differentiation or activation of Th1 and Th2 cells (*7*, *12*, *13*). Inhibition of the p38 MAP kinase pathway, either by the specific p38 inhibitor SB203580 or by the expression of dominant-negative p38 in transgenic mice, results in decreased production of IFN-γ in Th1 cells (*7*).

The inflammatory or immune response requires cell−cell interactions between leukocytes and targets cells (*14*). The formation of constitutive and inducible adhesion molecule complexes is necessary for the interaction of these cells. Differential expression of intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin gene superfamily, in the epidermis plays a critical role in the regulation of inflammation, immunologic reactions, and tissue repair (*15*, *16*). ICAM-1 mediates the firm binding of a variety of leukocytes to the target cells via its interaction with lymphocyte functional associated antigen 1 (LAF-1) or Mac-1 (CD11b/CD18) expressed on circulating white blood cells (*17*).

Using human peripheral blood lymphocytes we examined the effects of incubation with FIP-fve on cytokine secretion, induction of accessory molecule expression, and cellular proliferation. In parallel cultures we studied the effect of blocking MAPK on the T cell proliferation response and IFN-γ production to FIP-fve. Our results demonstrate that FIP-fve has potent effects on T cells. Coincubation of lymphocytes with FIP-fve resulted in enhanced ICAM-1 expression concomitant with elaboration of cytokine, IFN-γ, and cellular proliferation. The PBMCs activated by FIP-fve secrete significant amounts of IFN-γ but low detectable levels of IL-4, in vitro and in vivo. Here, we show that p38 MAP kinase is activated upon stimulation in Th1 cells, but not in Th2 cells, by FIP-fve.

MATERIALS AND METHODS

Materials. FIP-fve was purified as previously described (*1*). It resolved to a single band on SDS−15% PAGE with Coomassie brilliant blue R staining. SB203580 was obtained from Promega (Madison, WI). Phospho-ERK, ERK antibodies (Abs), phospho-p38, p38, and anti-rabbit IgG horseradish peroxidase- (HRP-) linked Ab used were all obtained from New England Biolabs (Beverly, MA).

Preparation of PBMC and Proliferation Assay. Human PBMCs were isolated from the heparinized peripheral blood of healthy adults by centrifugation over Ficoll-paque gradient medium (Amersham Biosciences). Cells (1×10^6 cells/mL) were cultured with or without stimulus in RPMI1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 units/mL penicillin, and 200 mM L-glutamate in 24-well tissue culture plates (Nunc, Roskilde, Denmark) for 24, 48, and 72 h.

Cell Cycle. Cell cycle phases were analyzed on a fluorescence-activated cell sorter (FACScan, Becton-Dickinson, San Jose, CA) with ModFIT 3.0 software. For the determination of the cell cycle phases, a peak area of FL2-H was recorded on a linear scale. Aliquots of 1×10^6 cells were fixed with 70% ethanol overnight at 4 °C before centrifugation. The cell pellets were treated with propidium iodine (4 μg/mL) solution containing RNase (100 μg/mL) and Triton X-100 (1%) for 30 min. The stained cells were filtered through 70 μm nylon mesh and then applied on the FACScan.

Assay for Cytokines. For cytokine analysis, the cells (1×10^6 cells/mL) were plated into 24 well plates (Nunc, Roskilde, Denmark) in the presence of FIP-fve. Conditioned media from untreated or FIP-fve stimulated PBMC were assayed for the activity of IFN-γ and IL-4 by use of commercially available kits from R&D Systems (Minneapolis, MN), with a solid-phase enzyme-linked immunoabsorbent assay (ELISA) as described by the manufacturers. The amounts of ICAM-1 (intercellular adhesion molecule 1) were measured with commercially available kits from Cellfree (Pierce).

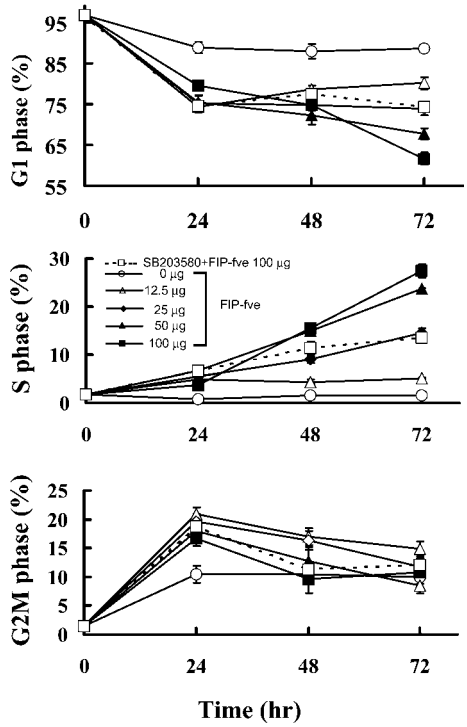

Figure 1. Cell cycle alterations measured as G0/G1 phase, S phase, and G2/M phase percentage obtained in hPBMC cells exposed to FIP-fve. Cultured peripheral T cells (2×10^6 cells/mL, 1 mL/well) were treated with the indicated concentrations of FIP-fve in RPMI 1640 supplemented with 5% FBS for various time period. Cell cycle phases (G1, S, and G2/M) were assessed by flow cytometry after 24, 48, and 72 h. Data are shown as percentage of nuclei in the sample and are representative of one of three experiments performed.

Western Blot Analysis. Cell lysates were resolved by SDS−PAGE before transfer to PVDF membrane via a transblot system (Hoffer Scientific mini VE). PVDF membranes were then incubated with blocking buffer (0.01 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.1% Tween 20) containing 3% bovine serum albumin (BSA) for at least 1 h to block nonspecific protein binding. Primary Abs were diluted in blocking buffer and applied to the filter overnight at 4 °C. Following washing, the blots were incubated with the appropriate HRP-conjugated secondary Ab (diluted up to 1:10 000 in blocking buffer for 1 h at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) system (NEN, Boston, MA).

Statistical Analysis. Cell cycle phases among each subgroup were analyzed by two-way ANOVA with Bonferroni multiple comparison testing. One-way ANOVA was used was to evaluate the IFN-$\gamma$and IL-4 production among each subgroup.

RESULTS

Cell Cycle Effect of SB203580 and FIP-fve Causes G1 to S Phase Proliferation. FIP-fve caused a detectable accumulation of nuclei in S phase with a concomitant decrease of nuclei in G1 phase that was most evident after 48 h and 72 h by two-way ANOVA with Bonferroni multiple comparison testing (**Figure 1**). After 72 h with 100 μg of FIP-fve stimulation, as demonstrated by staining cells with the DNA-specific fluorochrome PI, 61% of lymphocytes were identified as cells with a G0/G1 DNA content, while 26% and 12% were cells in S and

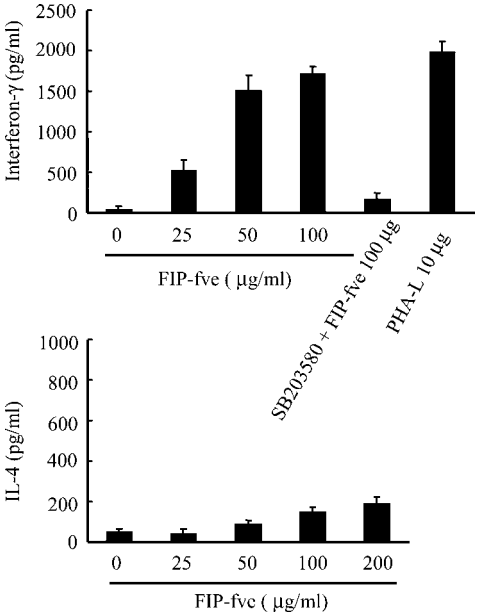

Figure 2. Effects of FIP-fve and PHA (10 μg) on IFN-γ and IL-4 secretion in PBMCs. (**A**) Effects of FIP-fve on IFN-γ secretion in PBMCs. Cultured peripheral T cells (2×10^6 cells/mL, 1 mL/well) were treated with the indicated concentrations of FIP-fve in RPMI 1640 supplemented with 5% FBS for 48 h. Cultured PBMC were pretreated with 10 μM SB203580 for 1 h and then in combination with 100 μg of FIP-fve. (**B**) Effects of FIP-fve on IL-4 secretion in PBMCs. Cultured peripheral T cells (2×10^6 cells/mL, 1 mL/well) were treated with the indicated concentrations of FIP-fve in RPMI 1640 supplemented with 5% FBS for 48 h. Conditioned media were subjected to ELISA to measure amounts of secreted IFN-γ and IL-4 (mean value from three independent experiments).

G2/M phases, respectively. In addition, it has also been reported that the ERK pathway is an important cellular signaling pathway that regulates cell proliferation. Indeed, we find that FIP-fve stimulates phospho-ERK (data not shown). In cultures treated with FIP-fve in the presence of 10 μM SB203580, the percentage of S cells was distinctly reduced and the percentage of G1 cells was increased compared to the cultures treated with FIP-fve alone. The degrees of reduction varied; with SB203580, more than 10% of cells were in S phase but the cell cycle cannot be fully repressed. Clearly, SB203580 partially prevented the lymphocytes' G1 to S phase progression from the cell cycle.

Effect of FIP-fve on Cytokine Production. To characterize the types of the functional T cells activated by FIP-fve, we analyzed the production of IL-4 and IFN-γ of human PBMCs by FIP-fve. **Figure 2** illustrates the production of IFN-γ and IL-4 by human PBMC with various concentration of FIP-fve. Untreated PBMCs secreted certain amounts of IFN-γ [45 pg (2×10^6 cells)$^{-1}$ mL^{-1}] and IL-4 [14 pg (2×10^6 cells)$^{-1}$ mL^{-1}]. After treatment with FIP-fve, the production of IFN-γ increased significantly in a dose-dependent manner and reached a plateau at 100 μg/mL. SB203580 (10 μM) could fully abolish the production of IFN-γ induced by FIP-fve. The production of IL-4 increased very slightly upon treatment with FIP-fve. To address the in vivo effects of FIP-fve, we injected FIP-fve into mouse peritoneum and subsequently analyzed the serum levels of IFN-γ and IL-4. As indicated in **Figure 5**, the considerable amounts of IFN-γ were detected after the third injection of FIP-fve. Under these experimental conditions, we were still not able to detect any significant amounts of IL-4 induced by FIP-fve. These results indicated that FIP-fve could preferentially activate IFN-γ-producing Th1 cells.

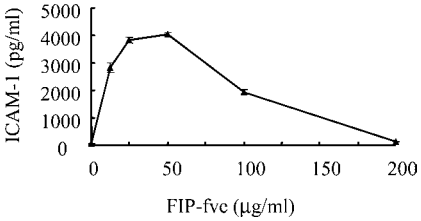

Figure 3. ICAM-1 is induced by various concentrations of FIP-fve in PBMCs: Effects of FIP-fve on ICAM-1 expression in PBMCs. Cultured peripheral T cells (2×10^6 cells/mL, 1 mL/well) were treated with the indicated concentrations of FIP-fve in RPMI 1640 supplemented with 5% FBS for 48 h.

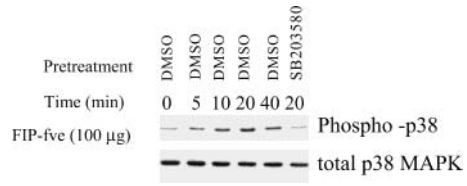

Figure 4. Activation of p38 and effect of SB203580 on p38 gene activation, analyzed by Western blot, in purified human PBMCs. Freshly purified PBMCs were stimulaed with 100 μg/mL FIP-fve for 5, 10, 20, and 40 min. Cell were pretreated with DMSO (0.05% v/v) or SB203580 (10 μM) for 30 min and then stimulated with FIP-fve for 20 min (right lane). Total cellular protein was analyzed by Western blot with pp38 antibody (upper panel) and reprobed with anti-total p38 antibody (lower panel).

FIP-fve-Induced Surface Expression of ICAM-1 As Determined by ELISA. In addition to cell proliferation and IFN-γ production, microscopic visualization of stimulated cells revealed that PBL cultured with FIP-fve formed large aggregates, whereas unstimulated cells remained in a single-cell suspension (data not shown). When the cells were examined for adhesion molecule expression, we found that FIP-fve was able to induce ICAM-1 expression of human PBMCs in a dose-dependent manner (**Figure 3**). FIP-fve-triggered T-cell responses were highly accessory cell-dependent, because T cells proliferated only with FIP-fve in the presence of adherent accessory cells. The molecular mechanisms as well as the role of accessory cells involved in the activation of particular T cell subsets are interesting and remain to be elucidated.

FIP-fve Activates p38 MAP Kinase in PBMCs. IFN-γ expression by Th1 effector T cells is mediated by the p38 MAP kinase signaling pathway (*7*). To investigate whether the p38 MAP kinase pathway is involved in FIP-fve signal transduction in PBMCs, we examined the activation of the MAP kinase by detecting its phosphorylated form by Western blotting using specific anti-phospho p38 kinase Ab (**Figure 4**). FIP-fve strongly stimulated a rapid (within 5 min) and transient increase in the levels of activation of p38 MAP kinase activity, which peaked at 20 min but remained elevated for at least 40 min. Little or no activation of p38 MAP kinase could be detected in unstimulated PBMCs. SB203580 appeared to suppress FIP-fve-stimulated p38 phosphorylation (**Figure 4**, lane 6).

DISCUSSION

We had extended a previous observation that FIP-fve is capable of inducing lymphocyte proliferation. Results of the present study demonstrate that FIP-fve is a potent activator of human peripheral blood lymphocytes. The binding of mitogens regulates several cytoplasmic signal transduction cascades, among which activation of the lectin-activated protein kinase cascade, the sequence of ras, Raf, MEK, and MAPK, is perhaps

best characterized (*18, 19*). FIP-fve induces cellular proliferation through the modulation of pERK. The ability of pERK to relinquish cellular growth arrest and promote G0/G1 to S phase transition likely contributes to its strong mitogenic potential. SB203580 did cause partial inhibition of T-cell proliferation during T-cell activation to FIP-fve, but it is unclear whether it was indirectly mediated by an effect of SB203580 on the antigen-presenting cells. p38 negatively regulates cyclin D1 expression, a G(1) cyclin required for cell cycle traversal (*20*). The interaction of ICAM-1 with its counterreceptor is an important mediator of cellular adhesion events in a broad spectrum of both antigen-dependent and antigen-independent interaction with immune cells, including the activation of T cells (*21*). The interactions of adhesion molecules with their respective ligands not only act to stabilize cell−cell contact but also send signals to the T-cell proliferative pathway induced by T-cell receptor/antigen/major histocompatibility complex (MHC) interactions. Results of this study demonstrate that FIP-fve initiates a cascade of events, which leads to upregulation of ICAM-1 expression on T cells.

The p38 mitogen-activated protein kinase (p38 MAPK) pathway is one of three distinct mammalian MAPK pathways that transduce a variety of extracellular (mainly stressful and inflammatory) signals and is activated by at least two specific MAPK kinases, MKK-3 and MKK-6 (*22*). Selective inhibition of this pathway can be achieved with pyrinidylimidazole compounds, which prevent activation of the downstream effector MAPK-activating protein kinase 2. The use of these highly specific inhibitors has shown that p38 MAPK is crucial for the production of Th1 cytokines, such as IFN-γ. We have utilized SB203580 in order to examine the effect of this pathway on the production of IFN-γ. Indeed, the treatment of FIP-fve induced the phosphorylation of p38 MAPK, which was significantly inhibited by 10 μM p38 MAPK, SB203580. It was demonstrated that the inhibition of IFN-γ production by Th1 cells caused by SB203580 is associated with reduced IFN-γ mRNA expression (*7*). Furthermore, they demonstrated that transcription mediated by the IFN-γ promoter is inhibited by treatment of cells with SB203580. Together, these data demonstrate that the p38 MAP kinase pathway regulates the expression of IFN-γ in Th1 cells to FIP-fve.

CD4+ Th2 cells play a pivotal role in initiating and orchestrating ongoing immunologically mediated allergic diseases. By contrast, CD4+ Th1 cells, which produce large amounts of IFN-γ, are able to inhibit the development of Th2 cells and IgE production (*23*). As shown in **Figure 2**, human PBMCs secreted significant amounts of IFN-γ after 48 h of FIP-fve activation. On the contrary, we did not detect any IL-4 production under the same conditions. In allergic patients, symptoms are mainly associated with high levels of allergen-specific IgE and eosinophils, which are induced by type 2 cytokines (such as IL-4 and IL-5) produced by Th2 cells (*24*). A delicate balance between IL-4 and IFN-γ production is required for the maintenance of normal IgE serum levels. We had previously demonstrated that the edema reaction and systemic anaphylaxis reaction could be prevented by repeat administration of FIP-fve, in vivo. This study is to characterize the immunomodulatory activity of FIP-fve. This study demonstrates that FIP-fve could selectively activate both human and murine interferon-γ-secreting Th1 cells. Therefore, stimulation of the predominant Th1 responses might be used as a potential candidate in modulating allergic immune responses.

We had observed highly similar amino acid sequences between FIP-fve and another immunomodulatory protein, Ling

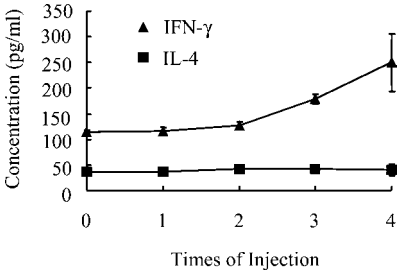

Figure 5. Serum levels of IL-4 and IFN-γ in mice after FIP-fve immunization in vivo. Mice were immunized with FIP-fve (10 mg/kg of body weight) through ip injections every other day. Serum were collected at 24 h after the last injection, and the amounts of IL-4 (■) and IFN-γ (▲) were measured by ELISA.

Zhi-8 (LZ-8) and FIP-GTS were isolated from *G. lucidium* and *Ganoderma tsugae*, respectively (*1, 25, 26*). There were 70 invariant amino acid residues (61.4%). LZ-8, a FIP, was found to have significant effects on cell immunity, such as preventing the occurrence of autoimmune diabetes mellitus in young female nonobese diabetic mice (*27*) and delaying the rejection of allografted mouse skin and transplanted allergenic rat pancreatic islets (*28*−*30*). FIP-fve exhibited potent mitogenic effects on human peripheral blood lymphocytes, inducing G1/G0 to S phase proliferation. The T cells activated by FIP-fve secrete a dramatic production of interferon-γ. Therefore, FIP-fve could be developed into new potential immunosuppressive agents. Peritoneal exudate cells that contained primarily macrophage were obtained in lavage of the peritoneal cavity. Upon stimulation by FIP-fve, macrophages might de novo synthesize and release a large variety of cytokines such as IFN-γ, but IL-4 (**Figure 5**) was not increased in serum. We suspected that the pleiotropic effect of IL-2 from macrophage activated T lymphocytes in splenocytes and consequently release of IFN-γ. In addition, many immunosuppressive agents, such as cyclosporin, prednisolone, and FK506, show toxic effects on pancreatic islets (*31*). Although the mice appeared to tolerate continuous treatments without symptoms of loss hair loss or piloerection, we had observed the spleen of animals treated with FIP-fve by ip injection is larger than that of control. The spleen is the largest of the secondary lymphoid organs and is highly efficient in trapping and concentrating foreign substances carried in the blood and the major organs in the body. The weight loss associated with ip FIP-fve was about 6.5%. Hematological analysis of mice was not significantly changed with the treatment (data not shown). Biochemical analysis of Balb/C mice showed some alteration in levels of GPT and Amyl. The toxicity of FIP-fve will further need to be clarified.

ABBREVIATIONS USED

ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FIP, fungal immunomodulatory protein; fve, *Flammulina velutipes*; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon-γ; IL, interleukin; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cells; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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