Induction and mode of action of the viral stress-inducible murine proteins, P56 and P54

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Abstract

Mammalian cells respond to virus infection or other viral stresses, such as double-stranded (ds) RNA and interferons (IFN), by robust and rapid induction of viral stress-inducible proteins. The induction and actions of one such protein, the human P56, have been extensively studied. However, little is known about the distantly related mouse proteins, MuP56 and MuP54. Here, we report that, in mouse cells, they could be induced by IFN, dsRNA or Sendai virus infection. MuP56 and MuP54 inhibited protein synthesis in vitro by binding to the “c”, but not the “e”, subunit of the translation initiation factor, eIF-3. The N-terminal region of the MuP54 was sufficient for inhibiting translation, but it and the corresponding region of MuP56 bound to two different regions of eIF3c. Thus, members of the human and murine P56 family have similar but non-identical functions.

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Introduction

Cells respond to virus infection in a variety of ways, one of which is by inducing transcription of cellular genes that are usually silent in uninfected cells (Sarkar and Sen, 2004). Induction of these viral stress-inducible genes (VSIG) can be mediated by a number of signaling pathways depending on the nature of the inducer (Sen and Sarkar, 2005). The inducer can be the infecting virions themselves (Zhu et al., 1998; Chang and Laimins, 2000; Johnston et al., 2001; Mossman et al., 2001), subviral particles derived from them (Boyle et al., 1999), viral gene products such as double-stranded (ds) RNA (Geiss et al., 2001) or single-stranded (ss) RNA (Lund et al., 2004) or virally induced cellular cytokines such as type I interferon (IFN) (Der et al., 1998; Geiss et al., 2003). Extensive analyses of human VSIG induction pathways have revealed that a large subset of these genes is induced through the interferon-stimulated response elements (ISRE) present in their promoters (Levy et al., 1968; Reich et al., 1987; Cohen et al., 1988; Porter et al., 1988; Pellegrini and Schindler, 1993; Bandyopadhyay et al., 1995). These elements bind members of the interferon-regulatory factor (IRF) family to promote transcription. IRF-3 and IRF-7 are the major members that get activated by various viral stresses, translocate from the cytoplasm to the nucleus and activate transcription of the target genes (Servant et al., 2002; Peters et al., 2002; Sato et al., 2000, Grandvaux et al., 2002). IFN, on the other hand, induces the same genes using the same cis-element, ISRE, but a different transcription factor, ISGF-3, that is composed of IRF-9, STAT1 and STAT2 (Darnell et al., 1994; Stark et al., 1998; Levy and Darnell, 2002). While the activation mechanism of ISGF-3 by IFN, using the Jak/STAT pathways, is well understood, the pathways used by various viral stresses to activate IRF-3 are still being delineated (Sharma et al., 2003). Among the major advances made recently in this endeavor is the identification of the key protein kinase, TBK-1, that phosphorylates IRF-3 (Fitzgerald et al., 2003;
McWhirter et al., 2004), the membrane-receptor, Toll-like receptor (TLR) 3 (Alexopoulou et al., 2001) and the soluble receptors, RIG-I and mDa 5 (Yoneyama et al., 2004; Andrejeva et al., 2004) of dsRNA, the adaptor protein TRIF (Yamamoto et al., 2002) and the membrane-receptor of single-stranded RNA, TLR7 and TLR8. Which of these proteins are used for activating IRF-3 depends on the specific virus and the host cell. Among the human VSIGs, the most prominent ones are the members of the ISG56 family (Der et al., 1998; Geiss et al., 2001). These genes are not expressed or expressed at a low level in most human cell lines, but they are induced to high levels upon infection with many viruses and treatment with IFN or dsRNA (Guo et al., 2000a). Human P56 interacts with the ‘c’ subunit of the eukaryotic translation initiation factor eIF3 (Guo et al., 2000b) and selectively inhibits its ability to stabilize the ternary complex of eIF-2, GTP and Met-tRNAi (Hui et al., 2003). As a result, human P56 inhibits initiation of translation. In human cells infected with hepatitis C virus, ISG56 is induced, and the encoded P56 protein is associated with ribosomes bound to HCV RNA, presumably causing a block in Hep C protein synthesis (Wang et al., 2003). The P56 family of proteins contains multiple tetratricopeptide repeat (TPR) motifs, which mediate protein–protein interactions (Sarkar and Sen, 2004). The interaction of human P56 with eIF3e is thought to be mediated by the PCI (named for proteasome, COP9/signalosome and initiation factor) (Hoffman and Bucher, 1998) motif of the latter protein (Guo et al., 2000b).

Little is known about the induction pattern of the P56 family of proteins in mouse. Recently, we have reported that the mouse p56 protein can also inhibit protein synthesis (Hui et al., 2005). But, in contrast to human P56, it interacts with, not the ‘e’, but the ‘c’ subunit of eIF3, thus underscoring key differences in the properties of human and mouse proteins. Here, we report the characteristics of induction of mouse P56 and mouse P54, in vitro, in response to dsRNA treatment and Sendai virus (SeV) infection. We also report that both proteins inhibit translation by interacting with eIF3c, but their interaction patterns are different, suggesting similar but not identical functions. The results reported here provide the groundwork for this important group of proteins in mouse, and the information provided by this study is essential for assessing their roles in mediating host response to infection by various viruses in a variety of mouse models.

Results

Comparisons of primary structures

Because human P56 and P54 have previously been identified as major viral stress-inducible proteins, we started an investigation of the corresponding mouse proteins, MuP56 and MuP54. MuP56 has only 53% sequence homology to HuP56, and the sequence is conserved mostly in the N-terminal half of the proteins (Fig. 1A). Similarly, MuP54 and HuP54 have only 62.8% sequence homology. There are even less sequence conservations between MuP56 and MuP54 as well as HuP56 and HuP54 (Fig. 1A). The only identified structural motifs present in HuP56 are six TPR motifs; MuP56 also has those six motifs (Fig. 1B). It is remarkable that these motifs are located at similar positions on the proteins, although their C-terminal halves have little sequence homologies. Both P54 proteins contain only four TPR motifs, all in the N-terminal parts of the proteins. The locations of the first TPRs are different in MuP54 and HuP54, but the other three are located in identical places. These analyses show that the TPR motifs, but not the overall sequences, are highly conserved between MuP56 and HuP56 and between MuP54 and HuP54.

Induction by IFN and dsRNA

Steady-state levels of the mRNAs for the murine genes encoding MuP56 and MuP54 were measured in primary mouse cells using quantitative RNase protection assays. In bone-marrow-derived macrophages (BMDM), there was no basal expression, but both mRNAs were strongly induced by either dsRNA or IFN-β (Fig. 2A). In contrast, in mouse embryo fibroblasts, only IFN-β could induce them (Fig. 2B). These results show that the MuP56 and the MuP54 genes can be induced by one agent, but not another, in different cell types. The observed differences could be...
attributed to the fact that IFN-β-receptor is expressed in both BMDM and MEF, whereas the dsRNA-receptor, TLR3, is expressed only in BMDM, but not in MEF.

To be able to examine the induction characteristics of the corresponding proteins, we attempted to raise anti-peptide antibodies for the two proteins. This was successful for MuP56, but not for MuP54. In Western blot analysis, the MuP56 antiserum recognized MuP56, expressed in cells by transfections, and the recognition was completely competed out by the peptide used for raising the antibody (Fig. 3A). In MEF, IFN-β induced MuP56 protein quite strongly (Fig. 3B), but as expected (Fig. 2B), dsRNA, when added to the culture medium, failed to induce MuP56 (Fig. 3C). But when dsRNA was transfected, MuP56 was strongly induced in MEF (Fig. 3C), demonstrating that activation of the internal RIG-I system by dsRNA leads to induction of MuP56 as well. In BMDM, as expected, both dsRNA and IFN-β could induce the protein, and the induction level peaked around 8 h (Fig. 3D).

**Induction by Sendai virus infection**

To examine whether MuP56 can be induced by virus infection, we infected mouse L929 cells with SeV. MuP56 was induced within 8 h after infection, and it was present in cells even 48 hpi (Fig. 4A) Similarly in MEF, SeV infection or IFN-β treatment induced the protein strongly (Fig. 4B).

To test whether the induction by SeV is mediated by intermediate production of IFN, we used Stat1−/− MEF. These cells cannot respond to any type of IFN because Stat1 is required for signaling by IFN-α, IFN-β and IFN-γ. In these cells, as expected, IFN-β could not induce MuP56 but SeV could, although with a slower kinetics (Fig. 4C). These results demonstrated that SeV infection could induce MuP56 directly. By inactivating the viral RNA genome using UV exposure of the virions, we investigated whether viral gene expression was required for MuP56 induction. UV-inactivated virus was equally capable of inducing MuP56 in mouse cells (Fig. 4D), suggesting that the infecting virions or subviral particles derived from them are capable of inducing these viral stress-inducible proteins.

**Inhibition of translation by MuP54 and MuP56**

In the next series of experiments, we investigated the functions of MuP54 and MuP56. For this purpose, the proteins were expressed in *E. coli* as hexahistidine-tagged proteins, and the recombinant proteins were purified by Ni-agarose chromatography (Fig. 5A). The abilities of the two proteins were determined by Western blot analysis. The specificity of the antibody is shown by blocking with the peptide against which the antibody was raised. A similar Western blot was performed with 60 μg of cell lysate from MEF treated with IFN-β (1000 U/ml) for 16 h. When dsRNA was added, the culture medium, failed to induce MuP56 (Fig. 3C). But when dsRNA was transfected, MuP56 was strongly induced in MEF (Fig. 3C), demonstrating that activation of the internal RIG-I system by dsRNA leads to induction of MuP56 as well. In BMDM, as expected, both dsRNA and IFN-β could induce the protein, and the induction level peaked around 8 h (Fig. 3D).

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murine proteins to inhibit protein synthesis were tested using an in vitro reticulocytes lysate system. Both of them inhibited translation almost as strongly as HuP56 (Fig. 5B). The inhibition by MuP54 was dose-dependent, and, at the highest doses tested, protein synthesis was reduced by almost 90% (Fig. 5C).

**Interaction with eIF3c**

HuP56 blocks initiation of protein synthesis by binding to the “c” subunit of the translation initiation factor eIF3 (Hui et al., 2003). The interaction between the two proteins can be detected by co-immunoprecipitation of epitope-tagged proteins expressed in mammalian cells. Using this assay, no interaction could be detected between MuP54 and eIF3c, although both proteins were expressed efficiently in the transfected cells (Fig. 6C). However, in the same experiment, as expected, a strong interaction was manifested between HuP56 and eIF3c. In contrast, MuP54 interacted with a different subunit of eIF3, eIF3c, as did MuP56 (Fig. 6D). Similarly, purified recombinant MuP54 interacted with purified eIF3c (Fig. 6B). Thus, both mouse proteins interact with an eIF3 subunit that is different from the one that interacts with human P56.

In the next series of experiments, we mapped the domains of MuP56 and MuP54 that interact with eIF3c. For this purpose, for both proteins, increasing deletion mutants from the C-terminal were expressed in cells, and their interactions with eIF3c were monitored by their co-immunoprecipitations. WT MuP56 and four deletion mutants were efficiently expressed, and they all interacted with eIF3c (Fig. 7A). Similarly, WT MuP54 and its three deletion mutants could all interact with eIF3c (Fig. 7B). These results demonstrated that N-terminal domains of MuP56, contain-

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Fig. 5. Recombinant MuP54 inhibits translation. (A) Purified His-tagged MuP56 and His-tagged MuP54 were detected by Coomassie blue staining. (B) Luciferase mRNA was translated using a rabbit reticulocyte lysate system containing [35S]cysteine in the presence of dialysis buffer or 0.9 μM purified MuP54, MuP56 or HuP56. Translated luciferase was analyzed by SDS-PAGE and quantitated using Molecular Diagnostic Phosphoimager. Inhibition is shown as a percentage of the dialysis buffer control (100% translation). (C) Different concentrations of purified MuP54 were tested for translation inhibition. Experiments were performed in triplicates.

Fig. 6. MuP56 and MuP54 interact with eIF3c, but not with eIF3e. (A and B) show in vitro binding experiments. 18 pmol His-MuP54 protein was incubated with 18 pmol of (A) purified eIF3 or (B) purified eIF3c. After incubations, the samples were pulled down with Ni⁺ beads, and the bound proteins were analyzed by Western blotting with antibody against (A) eIF3 or (B) flag. (C) and (D) show in vivo experiments. Expression vectors for (C) flag-tagged eIF3c or (D) flag-tagged eIF3c were co-transfected into HT1080 cells with expression vectors of myc-tagged MuP56 or MuP54, as indicated. Cell extracts were made, and immunoprecipitations (IP) were performed with Flag antibody-conjugated agarose beads followed by Western blot (wb) with anti-Myc or anti-Flag antibody, as indicated. Cell lysates (60 μg) were also directly subjected to Western blotting (wb) with anti-Flag or anti-Myc antibody.
ing TPR1 and TPR2, and of MuP54, containing only TPR1, are sufficient for binding to eIF3c (Fig. 7C). To map the regions of eIF3c that interact with MuP54 and MuP56, we generated eIF3c*, a C-terminal deletion mutant of eIF3c. This mutant interacted with MuP56 (Fig. 8A), but not with MuP54 (Fig. 8B), although the wt protein interacted with both. These results suggest that the N-terminal of MuP56 interacts with the C-terminal region of eIF3c, whereas the N-terminal region of MuP56 interacts with the N-terminal region of eIF3c (Fig. 8C).

To test the functional capacity of the N-terminal domain of MuP54, a truncated protein (d1) containing only residues 1–127 aa was expressed in bacteria and purified by affinity chromatography (Fig. 9A). As expected, MuP54-d1 interacted with purified eIF3c* (Fig. 9B) and with purified eIF3c (Fig. 9C). It also inhibited translation in vitro in a dose-dependent fashion (Fig. 9D). Even though the truncated protein, d1, was about 8-fold less active, as compared to the full-length protein, at the highest dose tested (60 μM), it inhibited protein synthesis by more than 90%. Thus, the observed physiological interaction between d1 and eIF3 resulted in a strong inhibition of eIF3 function.

**Discussion**

The mouse ISG56 family has three members, ISG56, ISG54 and ISG49 encoding proteins of calculated molecular masses of 53.7 kDa, 55.0 kDa and 47.2 kDa. Phylogenetic analysis shows that MuP56 protein is most closely related to HuP56, MuP54 to HuP54 and MuP49 to HuP60 (Sarkar and Sen, 2004). The MuISG56 and MuISG54 genes have similar organizations, and both are induced by IFN-α (Blyussken et al., 1994). The promoter of MuISG54 has one authentic IFN-stimulated response element (ISRE), whereas the MuISG56 promoter has two such elements. Since these elements can be recognized by many members of the IRF family of transcription factors, a variety of viral stresses that activate different signaling pathways leading to IRF activation may be able to induce transcription of these genes. The relevant mediators of viral stress include type I IFNs, dsRNA and viral ribonucleoproteins.

As expected, both MuP56 and MuP54 mRNAs were induced in BMDM and MEF by IFN-β. They were also induced by dsRNA in BMDM, but not in MEF cells (Fig. 2). Because BMDM, but not MEF cells, express TLR3, a known
mediator of extracellular dsRNA signaling, the induction of these genes by dsRNA was most probably mediated by TLR3. Activation of the intracellular RIG-I pathway by transfection of MEF with dsRNA also caused activation of the MuP56 gene. An antibody was raised, and we confirmed induction of the protein in MEF by IFN-α and in BMDM by dsRNA and IFN-α (Fig. 3). The protein was detected as early as 4 h and was present in abundant quantity even 24 h after dsRNA treatment. This is the first demonstration of induction of MuP56 because no antibody was previously available. A third agent, SeV, also induced the protein efficiently (Fig. 4). Viral induction of the protein did not require viral gene expression because UV-inactivated SeV was equally effective (Collins et al., 2004). Because viral induction of MuP56 was observed in STAT1−/− MEF, it does not require the action of IFN as an intermediate. However, the induction was quicker and more robust in Wt cells (compare Figs. 4B and C), indicating that both IFN-dependent and independent pathways may contribute to full induction of this protein in response to SeV infection. The viral induction does not require TLR3 because MEFs, which do not express TLR3, responded to SeV but not to dsRNA. This observation is consistent with our recent observation of TLR3-independent induction of HuP56 in response to SeV infection (Elco et al., 2005).

The second part of this study was devoted to examining the function of MuP56 and MuP54. For designing these experiments, we used our experience with HuP56 (Guo et al., 2000b). Like HuP56, recombinant MuP56 and MuP54 inhibited translation in vitro, and on a molar basis, the three proteins were similarly effective (Fig. 5). Although all three proteins interacted with the translation initiation factor eIF3, the interaction was mediated through different subunits. While HuP56 interacted with eIF3e, both murine proteins interacted with eIF3c but not with eIF3e (Fig. 6). This reciprocal specificity was unexpected especially between HuP56 and MuP56 because of their similarity in sequence and in the distribution of TPR motif (Fig. 1). The eIF3c-interacting domain of MuP56 and MuP54 were mapped to the N-terminal one-third of the proteins. For MuP56, this region contains two TPR motifs, whereas for MuP54, this region contains only one TPR motif (Fig. 1A). The difference in their sequences was reflected in the functional disparity of their interactions with eIF3c: MuP56 interacted with the N-terminal region of eIF3c, whereas MuP54 did not.

The eIF3c-interacting region of MuP54 (d1) was expressed in E. coli and purified to homogeneity. Unfortunately, the corresponding domain of MuP56 (d1*) could not be expressed in a soluble form. As expected, d1 could inhibit translation in vitro, thus functionally validating our interaction domain mapping results. On a molar basis, d1 was about 8 times less effective than the full-length MuP54. This difference could be due to weaker binding of d1 to eIF3c or less efficient folding of recombinant d1. We have recently reported that MuP56 inhibits a distinct function of eIF3, namely, its ability to promote formation of an initiation complex of 40s ribosome subunit, mRNA and the eIF2-GTP·tRNAMet ternary complex (Hui et al., 2005). In contrast, HuP56 inhibits a different function of eIF3 in stabilizing formation of the ternary complex (Hui et al., 2003). The specific function of eIF3 impaired by MuP54 has not been identified yet. Our observation that MuP56 and MuP54 bind to different regions of eIF3c suggests that they may inhibit different functions of eIF3 (Hershey and Merrick, 2000; Benne and Hershey, 1976; Merrick et al., 1973; Chaudhuri et al., 1999; Gupta et al., 1990; Imataka and Sonenberg, 1997; Lamphear et al., 1995) which, in turn,
may affect translation of different mRNAs to different degrees.

Although the human P56 family of proteins is new and widely recognized as the premier marker of viral stresses, the corresponding mouse proteins have not been studied before. Thus, this report provides the first characterization of mouse P56 and P54 induction and functions and describes assays and reagents that will be useful for future studies. For the mode of induction studies, the mouse system provides the strength of using knock-out mice and cells for various receptors and signaling proteins. For the functional aspects, induction and function of MuP56 and MuP54 in various mouse models of viral pathogenesis can be studied. The studies shown here can shed light on the specific functions of these proteins in vivo, their tissue distributions and cell type specificity. Similar experiments will be difficult or impossible to perform in the human system.

Materials and methods

**Cells, cell treatments and infection**

HT1080 human fibrosarcoma (Leonard and Sen, 1997), L929 murine fibroblast, mouse embryonic fibroblast (MEF) WT and Stat1−/− (Qing and Stark, 2004) were all maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary macrophages derived from bone marrow (BMMs) were isolated from the femur of FVB mouse (Whitmore et al., 2004).

HT1080 cells were transfected using Eugene 6 (Boehringer Mannheim Co.). After 18 h, cells were collected, and lysates were used for standard Western blotting or for immunoprecipitation. Murine IFN-β 1000 U/ml (Calbiochem) and dsRNA [poly(I)–poly(C)] 100 μg/ml (Pharmacia) were added to the culture medium, and the cells were treated for the desired time periods as indicated in the figures. MEF cells were transfected with Eugene 6 alone or with Eugene 6 and dsRNA (2 μg/ml). After 16 h, cells were lysed, and Western blots were performed using polyclonal antibody against MuP56. Cells (1 × 10⁶) were infected with Sendai virus (Sendai/Cantell, ATCC VR-907) at 80 HAU/ml for 1 h in serum-free DMEM. Medium was removed, and the cells were washed then refeed with DMEM which contained 10% serum and incubated at the time periods indicated in the figures. UV treatment of virus was performed according to a reported protocol (TenOever et al., 2002).

**Antibody**

Polyclonal antibody, anti-MuP56, was raised by Biosynthesis Incorporated, Lewisville, TX, against a peptide encoding amino acids 217–233 of MuP56 protein. Serum from injected rabbits was collected and tested for their anti-MuP56 activity by Western blot. The antibody specificity was studied by competing with excess of antigen. Briefly, 1 μl of antibody was first incubated with different concentrations of peptide (as indicated in the figures) for 2 h at 37 °C, then 16 h at 4 °C. The tubes were centrifuged for 15 min at 14,000 rpm at 4 °C, and the supernatant was used for Western blotting.

**Constructs**

The full-length of eIF3c was constructed by RT-PCR, the cDNA sequences were inserted into pFLAG-CMV-2 (Kodak Scientific Imaging System) containing Flag on the N-terminal domain. MuP56 full length was generated by PCR using an existing clone (our unpublished data), the cDNA sequence was subcloned in Myc-pcDNA3. This vector was generated inserting six repeat of 30 nt of the c-myc peptide in the N-terminal domain of pcDNA3 expression vector. MuP54 or MuP56 deletion mutants were generated by PCR and subcloned into Myc-pcDNA3. The full-length eIF3e was described previously (Guo et al., 2000b). The myc-MuP54 plasmid was a generous gift from Dr. David E. Levy.

**RNase protection assay**

RNA was isolated using RNAzol B reagent according to the manufacturer’s protocol (Teltest, Friendswood, TX). RNase protection assays (RPAs) were performed with the RPA III kit (Ambion, Austin, TX) following the manufacturer’s protocol. The antisense probes to MuP56 and MuP54 were generated first by cutting the cDNAs with MboII and StuI, respectively, and then transcribing both with SP6 RNA polymerase. For each sample, 20 μg of total RNA was used for RPA, protected mRNA levels were visualized by autoradiography.

**In vitro translation inhibition assay**

A 0.5 μg aliquot of luciferase mRNA (Promega) was added to 25 μl reaction of a rabbit reticulocyte lysate in vitro translation reaction (Promega) in the presence of recombinant purified protein, as indicated in the figure legend. The in vitro translations were performed under conditions recommended by the manufacturer, and the reaction mixture was incubated at 30 °C for 2 h. Newly synthesized [35S]-labeled proteins were analyzed by loading 5 μl of reaction on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by a phosphorimagery using Molecular Dynamics ImageQuant Software.

**Purification of recombinant protein from E. coli**

MuP54 and MuP56 were subcloned in pET-15b vector (Novagen). The proteins were expressed in bacterial strain BL21-DE3 pLys (Novagen) by induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 12 h at 30
Acknowledgments

Immunoprecipitation and Western blotting

Immunoprecipitation of Flag-tagged protein was performed in low salt buffer [20 mM Tris pH 7.5, 50 mM KCl, 200 mM NaCl, 1 mM EDTA, 20% glycerol, 0.05% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. The M2 anti-Flag agarose beads (Sigma) were pre-soaked with 3 ml of wash buffer I [500 mM KCl, 20 mM Tris pH 7.9, 10% glycerol, 35 mM imidazole, 5 mM β-mercaptoethanol, protease inhibitor] and 30 ml of washing buffer II [500 mM KCl, 20 mM Tris pH 7.9, 10% glycerol, 35 mM imidazole, 5 mM β-mercaptoethanol, protease inhibitor]. Bound His-MuP56 or His-MuP54 were eluted with elution buffer [500 mM KCl, 20 mM Tris pH 7.9, 10% glycerol, 35 mM imidazole, 5 mM β-mercaptoethanol, protease inhibitor] and dialyzed against a high salt buffer [150 mM KCl, 20 mM Tris pH 7.9, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] The whole-cell extract was mixed with 500 µl of low salt buffer and subjected to denaturating gel electrophoresis overnight. The immunocomplexes were washed with the low salt buffer and resuspended and sonicated in lysis buffer [500 mM KCl, 20 mM Tris pH 7.9, 10% glycerol, 5 mM β-mercaptoethanol and protease inhibitor].


