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LysRS Serves as a Key Signaling Molecule in the Immune Response by Regulating Gene Expression

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SUMMARY

Lysyl-tRNA synthetase (LysRS) was found to produce diadenosine tetraphosphate (Ap_4A) in vitro more than two decades ago. Here, we used LysRS silencing in mast cells in combination with transfected normal and mutated LysRS to demonstrate in vivo the critical role played by LysRS in the production of Ap_4A in response to immunological challenge. Upon such challenge, LysRS was phosphorylated on serine 207 in a MAPK-dependent manner, released from the multisynthetase complex, and translocated into the nucleus. We previously demonstrated that LysRS forms a complex with MITF and its repressor Hint-1, which is released from the complex by its binding to Ap_4A, enabling MITF to transcribe its target genes. Here, silencing LysRS led to reduced Ap_4A production in immunologically activated cells, which resulted in a lower level of MITF inducible genes. Our data demonstrate that specific LysRS serine 207 phosphorylation regulates Ap_4A production in immunologically stimulated mast cells, thus implying that LysRS is a key mediator in gene regulation.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) are extremely conserved during evolution. AaRSs play a central role in the translation of the language of nucleotides into the amino acid sequence of protein. This reaction occurs in the cytoplasm of all living cells (Ibba and Soll, 2000). Several studies have revealed that in addition to the well-known role in the catalysis of amino acid to the cognate tRNA, many of the aaRS have noncanonical roles (Szymanski et al., 2000). For instance, glutamyl-prolyl-tRNA synthetase (GluProRS) is involved in gene-specific silencing of translation (Sampath et al., 2004). Another example was observed with tyrosyl-tRNA synthetase, which was secreted by the cells during apoptosis and acquired cytokine activities (Wakasugi and Schimmel, 1999a, 1999b).

Lysyl-tRNA synthetase (LysRS) was previously shown to be localized in the cytoplasmic department (Gunasekera et al., 2004) and can be found in mammalian cells mostly in multisynthetase complex (MSC) (Bandyopadhyay and Deutscher, 1971), which contains three nonenzymatic proteins, p43, p18, and p38, and nine different aaRSs. Many of these aaRSs have been found to be involved in different signaling pathways; therefore, the MSC was termed "signalosome." While several aaRSs have been demonstrated to be able to produce diadenosine tetraphosphate (Ap_4A), which is composed of two adenosine moieties joined in 5′-5′ linkage by a chain of four phosphates, it has been reported that LysRS is the major contributor to the production of this nucleotide (Wahab and Yang, 1985). Moreover, the free form of LysRS from rat liver was reported to synthesize higher levels of Ap_4A than the form associated to the MSC (Wahab and Yang, 1985). Several studies have demonstrated that the concentrations of Ap_4A increase after exposure of cells to various forms of metabolic stress (heat, oxidative, nutritional, and DNA damage). For that reason they have been described as "alarmones" in cellular and metabolic stress both in prokaryotes (Lee et al., 1983) and in eukaryotes (Varshavsky, 1983).

These studies implicated a crucial role for Ap_4A in the regulation of the cellular response to various stresses (reviewed by Kisselev et al., 1998). Additionally, in vitro studies show that phosphorylation of aaRS does not affect the aminoacylation reaction but increases Ap_4A production by up to 6-fold (Dang and Traugh, 1989). None of these studies, however, was followed by any systematic attempts to reveal the regulatory mechanisms of Ap_4A production.

We have been studying the regulation of MITF and USF2 for over a decade (Levy et al., 2002; Nechushtan and Razin, 1998, 2002; Sonnenblick et al., 2004). During our studies, we discovered that LysRS forms a complex with each of these

transcription factors and hypothesized that this association is not related to the well-known role of LysRS as a tRNA synthetase, but to one of its "moonlight" functions as a producer of Ap_4A (Lee et al., 2004; Lee and Razin, 2005). Furthermore, we demonstrated by utilizing either external introduction of Ap_4A or silencing of the endogenous Ap_4A hydrolase (Carmi-Levy et al., 2008) that increased levels of Ap_4A are associated with increased MITF and USF2 transcriptional activity.

Here, we propose a signaling pathway in which phosphorylation of LysRS on a specific serine residue affects its dissociation from the MSC and enhances its capability to synthesize Ap_4A and, therefore, to regulate MITF gene expression.

RESULTS

The Induction of Ap_4A Synthesis in IgE-Ag-Activated RBL Cells Is Mediated by LysRS

To determine whether, indeed, LysRS is responsible for the level of Ap_4A in mast cells, LysRS was knocked down in a rat basophilic cell line (RBL) using the short interfering RNA (siRNA) approach. This siRNA was designed to be complementary to nucleotide sequences found in rat mRNA of LysRS, and a nonrelevant nucleotide sequence was used as the control (NR siRNA). Downregulation of the LysRS protein was observed 24 hr after transfection of the RBL cells with siRNA, and the levels of the protein remained low for up to 48 hr (Figure 1A). There was almost no Ap_4A accumulation in LysRS siRNA-transfected IgE-Ag activated RBL cells, whereas its level in the NR siRNA treated cells was similar to that in activated cells without transfection (Figure 1B). These results are complementary to our previous findings that Ap_4A levels were increased in cells overexpressing LysRS (Lee and Razin, 2005).

The effect of LysRS siRNA on total protein synthesis was determined by introducing ^{35}[S]-methionine into cells 24 hr after the administration of siRNA against LysRS. No significant change in the total cellular protein synthesis was observed in the LysRS knockdown cells compared to controls (Figure S1 available online).

FcεRI Aggregation Induces the Release of LysRS from the MSC

As mentioned above, LysRS is usually found in mammalian cells as a part of the MSC; however, in eukaryotic cells, it is several-fold more efficient at producing Ap_4A when found dissociated from the MSC. Thus, we examined whether LysRS molecules are released from the MSC upon immunological activation of RBL cells. In order to assess this dissociation, gel filtration chromatography of protein extracts derived from activated or nonactivated RBL cells was performed. As shown in Figure 2A, in nonactivated RBL cells, LysRS was mostly associated with the MSC. However, following activation, a shift of the LysRS molecules into the low molecular weight (MW) fractions was observed. Thus, LysRS molecules dissociated from the MSC upon mast-cell activation by FcεRI aggregation.

The dissociation of other proteins from the MSC (p43, ArgRS, and MetRS) was examined after cells were cultured with IgE-Ag (Figure S2). As opposed to LysRS, either minor or no dissociation

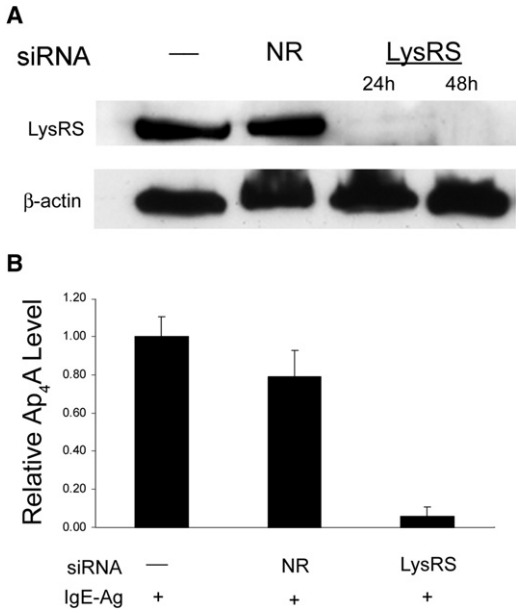

Figure 1. Silencing of LysRS Results in Abrogation of Immune Induction of Ap_4A

(A) RBL cells were transfected with siRNA, and whole-cell proteins were extracted 24 hr and 48 hr after the transfection. The level of LysRS was determined by western blotting analysis using antibody against LysRS. One representative experiment out of three is shown.

(B) Ap_4A levels were determined in RBL cells transfected with siRNA against LysRS and with NR siRNA. After 24 hr, the cells were activated with 100 ng/ml IgE anti-DNP and 100 ng/ml DNP for 15 min. The mean and standard error of the mean (SEM) of three experiments is shown.

was observed from the MSC, though the complex seems to be of reduced molecular size after the stimulus.

We subsequently determined whether the dissociation from the MSC was a result of a posttranslational modification, such as a rapid phosphorylation. We found an indication for this hypothesis, as in vitro studies have shown that phosphorylation of aaRSs does not affect the aminoacylation reaction but increases Ap_4A production by up to 6-fold (Dang and Traugh, 1989). Immunoprecipitation of potential phosphorylated proteins with phosphospecific antibodies and immunoblot analysis with anti-LysRS demonstrated phosphorylation of LysRS on serine (Figure 2B), but not on threonine residues after cell activation (Figure S3).

The MAPK pathway is one of the most important mast-cell pathways involved in signaling via FcεRI aggregation stimuli (Furuno et al., 2001; Santini and Beaven, 1993; Tsai et al., 1993). A key enzyme in this pathway is MAPK/ERK kinase (MEK). To determine whether LysRS phosphorylation is mediated by MEK, cells were treated with IgE followed by 10 μM U0126, a known, specific inhibitor of MEK (Bain et al., 2007; DeSilva et al., 1998), and then activated with antigen for 30 min. The results clearly show that this inhibitor blocked the serine phosphorylation of LysRS (Figure 2C).

In addition to the immunoprecipitation experiment, we used 2D gel electrophoresis in order to further confirm the

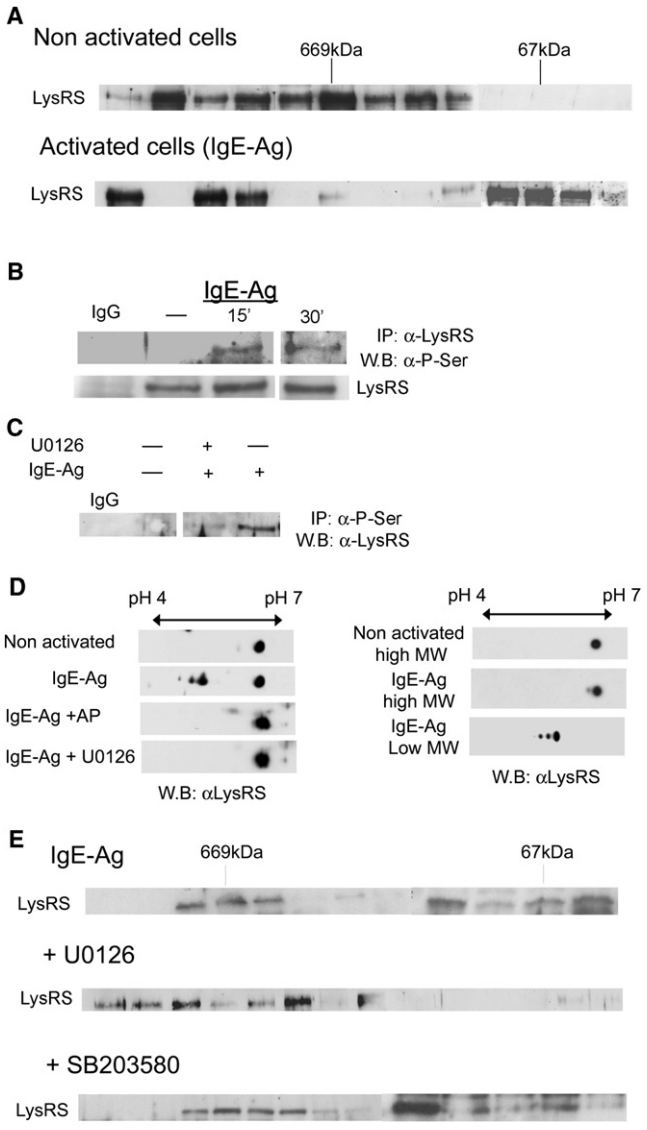

Figure 2. Mast Cell IgE-Ag Stimulation Induces LysRS MAPK-Dependent Serine Phosphorylation and Release from the Multisynthetase Complex

(A) Lysates from RBL cells that were either immunologically activated for 30 min or nonactivated were subjected to size-exclusion chromatography. Two milliliter fractions were collected. The eluted proteins in each fraction were analyzed by immunoblotting with anti-LysRS. One representative experiment out of three is shown.

(B) Serine phosphorylation of LysRS in mast cells is mediated by FcεRI activation. Lysates from cells treated with IgE and antigen for 15 or 30 min were immunoprecipitated (IP) with anti-LysRS antibody and subjected to western blot analysis (WB) with antibody against anti-phosphorylated-serine (P-Ser). One representative experiment out of three is shown.

(C) Lysates from cells treated with IgE-Ag alone (control) or with IgE followed by 10 μM U0126 for 10 min and then activated with antigen for 30 min, were immunoprecipitated (IP) with anti-P Ser and subjected to western blot analysis (WB) with antibodies against LysRS. One representative experiment out of three is shown.

(D) Lysates from RBL cells treated with IgE-Ag alone, or with IgE followed by 10 μM U0126 for 10 min and then activated with antigen for 30 min, were subjected to 2D electrophoresis on a pH 4–7 gradient and a 8% polyacrilammide gel. The gel was blotted with anti-LysRS antibody.

(E) Lysates from RBL cells that were stimulated without kinase inhibitors (upper panel), with U0126 (10 μM) for 10 min (second panel), or with SB203580 (5 μM) for 2 hr (lower panel) were subjected to size-exclusion chromatography. The eluted proteins in each fraction were analyzed by immunoblotting with anti-LysRS. One representative experiment out of three is shown.

phosphorylation of LysRS upon IgE-Ag activation. It should be noted that the antibodies anti-LysRS and anti-Myc reacted at the expected molecular weight of either rat LysRS or Myc-human LysRS with calculated pIs of 6.16 and 5.77, respectively.

Phosphorylation of LysRS was not detected in unstimulated cells, whereas in cells activated with IgE-Ag, spots shifted to the left side of the gel, indicating a more acidic pI compatible with phosphorylation. Moreover, this phosphorylation was specifically found in the low MW fractions, that is, the free form of LysRS (Figure 2D). This phosphorylation was totally blocked by the presence of alkaline phosphatase (AP) and U0126, which, in accordance to the immunoprecipitation results, indicates the involvement of MAPK in LysRS phosphorylation.

Next, proteins were extracted from cells triggered by FcεRI aggregation due to 30 min antigen activation, and these extracts were subjected to gel filtration chromatography. As shown in Figure 2E, the use of MEK inhibitor (U0126) completely prevented the release of LysRS from the MSC (second panel), whereas the p38 MAPK inhibitor, SB203580 (Bain et al., 2007), did not effect the dissociation of LysRS from the MSC (lower panel). Thus, the release of LysRS from the MSC was dependent on phosphorylation of its serine residues via MEK.

Serine 207 Phosphorylated LysRS Is Required for Ap_4A Synthesis

The involvement of the MAPK pathway in LysRS activity was further investigated by searching for ERK consensus motifs (X-S/T-P) within the LysRS sequence using multiple sequence alignment (Figure S4). Two serine residues within an ERK consensus motif in LysRS were detected (S207 and S470). Based on this, four fused Myc tag constructs of wild-type and mutated human LysRS (hLysRS) were constructed (WT, S207A, S470A, and S207A/S470A). Each one of the constructs was administered to the cells after the endogenous LysRS was knocked down by siRNA. Under these conditions, no gene silencing of the exogenous hLysRS occurred (Figure 3A). The Ap_4A assay was carried out on extracts derived from immunologically activated RBL cells that were transfected with each of the constructs. As can be seen in Figure 3B, mutation S207A of hLysRS significantly reduced Ap_4A production in these transfected RBL cells, similar to cells transfected with rat LysRS siRNA. Identical results were obtained for the cells transfected with the double mutation (S207A/S470A). Ap_4A accumulation was not affected as a result of the transfection of RBL cells with the S470A mutation. These observations clearly demonstrated that phosphorylation of serine 207, which is within the ERK consensus motif, is required for the synthesis of Ap_4A by LysRS.

Since phosphorylation of LysRS at Ser207 was found to have a major effect on Ap_4A synthesis, we next analyzed the

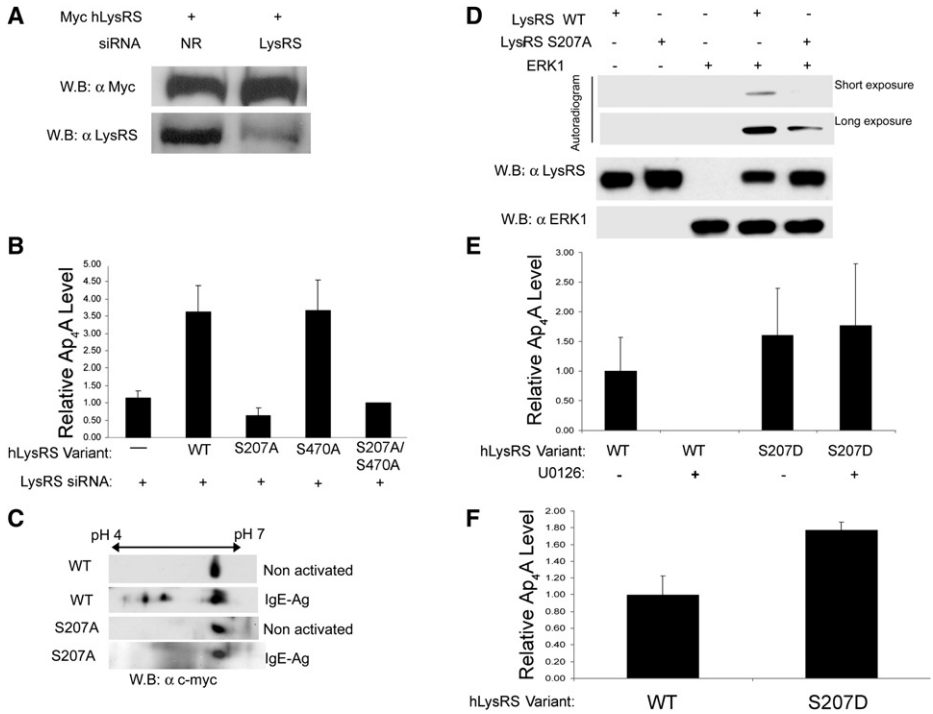

Figure 3. Serine 207 Phosphorylated LysRS Is Required for Ap_4A Synthesis

(A) RBL cells were transfected with rat LysRS siRNA (as described in the Experimental Procedures). Twenty-four hours later, cells were transfected with human LysRS variants (WT, S207A, S470A, or S207A/S470A). Next, the cells were incubated with IgE and challenged with DNP for 30 min. The cell extracts were analyzed by western blot with anti-Myc or anti-LysRS antibodies. One representative experiment out of three is shown.

(B) RBL cells were transfected and activated as described above. The Ap_4A assay was performed on the cells extracts as described in the Experimental Procedures. The mean and standard error of the mean (SEM) of three experiments is shown.

(C) Lysates from IgE-Ag-activated and -nonactivated RBL cells transfected either with WT or with S207A LysRS variants were subjected to 2D electrophoresis on a pH 4–7 gradient and a 8% polyacrilammide gel. The gel was blotted with anti-myc antibody.

(D) Recombinant LysRS proteins (WT/S207A) were expressed as His fusion proteins then phosphorylated in vitro in the presence of recombinant, active ERK1 and γ^{32}P-ATP. Samples were resolved on SDS-polyacrylamide gel and blotted, and the radioactive bands were detected by autoradiography.

(E) Ap_4A levels were determined in RBL cells transfected with either WT or S207D LysRS variants, cultured with or without U0126 (10 μM) for 10 min, and then activated with antigen for 15 min. The mean and standard error of the mean (SEM) of three experiments is shown.

(F) Ap_4A levels were determined in quiescent RBL cells transfected with either WT or S207D LysRS variants. The mean and standard error of the mean (SEM) of three experiments is shown.

phosphorylation status of LysRS WT and S207A variants upon immunological activation of mast cells. Strikingly, while the WT variant, similarly to the endogenous LysRS in Figure 2D, was shown to shift to the acidic side of the gel following IgE-Ag stimulation, the S207A variant was not affected by the trigger (Figure 3C).

When recombinant LysRS proteins (WT/S207A) were incubated with [γ-^{32}P]ATP in the presence of recombinant ERK1 and analyzed via SDS-PAGE, LysRS WT was clearly phosphorylated by ERK1, whereas LysRS S207A phosphorylation seemed significantly reduced (Figure 3D). Using densitometry analysis, the observed reduction in the phosphorylation was found to be 62%.

In order to further establish phosphorylation of Ser207 by MAPK as a crucial step toward Ap_4A production, we transfected cells with either LysRS WT or LysRS S207D variants. Ap_4A synthesis in cells transfected with the LysRS S207D variant was found to be insensitive to the MEK inhibitor U0126 (Figure 3E). Moreover, the S207D variant was shown to have constitutive Ap_4A production activity even in quiescent RBL cells when compared to WT (Figure 3F).

IgE-Ag Activation, via MAPK Pathway in RBL Cells, Induces Translocation of LysRS from the Cytosol into the Nucleus

LysRS has been identified as an MITF-interacting protein, using a construct containing the MITF bHLH-Zip domain as bait in yeast two-hybrid library screening (Razin et al., 1999). This protein-protein interaction within the complex was later verified by GST pull-down assays and coimmunoprecipitations (Lee et al., 2004; Nechushtan and Razin, 2002). Since MITF transcriptional activity is compartmentalized to the nucleus, we examined whether aggregation of FcεRI by IgE-Ag that induced the release of LysRS from the MSC (as we described above) also causes translocation of LysRS from the cytosol to the nuclear compartment. Using immunofluorescent labeling of LysRS, we found that nuclear translocation occurred 30 min after cell activation by IgE-Ag (Figure 4A). Confirmation of these findings was carried

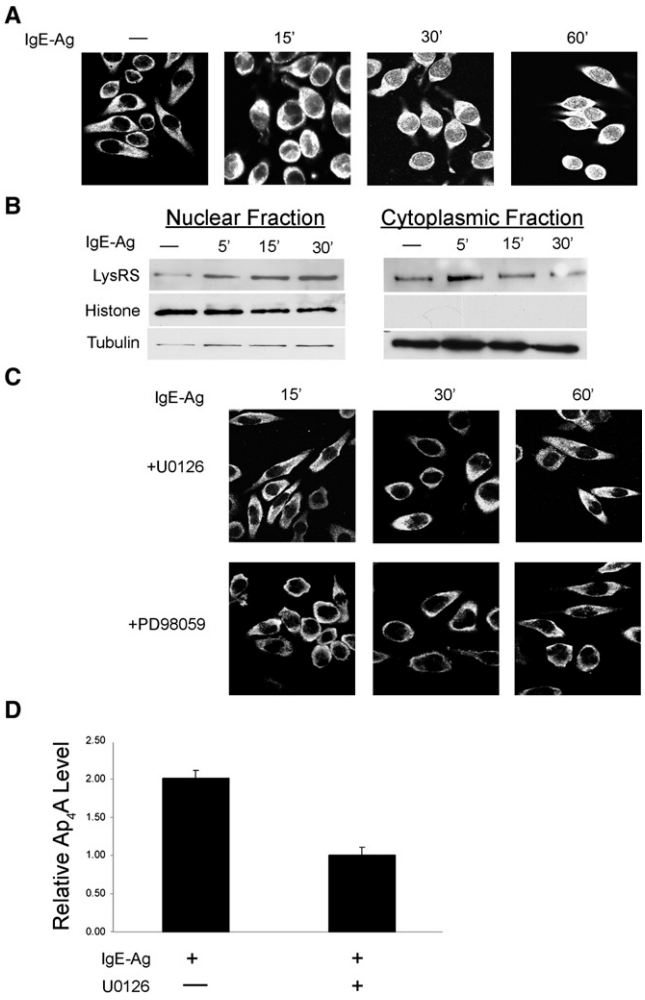

Figure 4. Identification of LysRS Nuclear Translocation in RBL Cells following FcεRI Aggregation

(A) RBL cells were sensitized with IgE and then were challenged with antigen for 15, 30, and 60 min. Immunostaining was performed with anti-LysRS using Cy5-labeled secondary antibody. The cells were analyzed by confocal laser scanning microscopy. Data shown are representative of three independent experiments.

(B) Nuclear and cytoplasmic fractions were isolated from RBL cells activated with IgE and antigen. The subcellular extracts were analyzed by western blot with anti-LysRS antibodiy. Antitubulin was used as a cytoplasmic marker while antihistone was used as nuclear marker. One of three independent experiments is shown.

(C) RBL cells were pretreated with IgE and with either MAPK inhibitor U0126 (10 μM, 10 min; top) or PD98059 (50 μM, 30 min; second line) and then were challenged with antigen for 15, 30, and 60 min. Immunostaining was performed with anti-LysRS using Cy5-labeled secondary antibody. The cells were analyzed by confocal laser scanning microscopy. One of three independent experiments is shown.

(D) RBL cells were activated for 15 min by IgE-Ag with or without U0126 treatment. Ap_4A levels were measured as described above. The mean and standard error of the mean (SEM) of three experiments is shown.

out by western blot analysis of the nuclear and cytosolic fractions with anti-LysRS (Figure 4B).

We then examined the effect of U0126 and PD 098059, another specific inhibitor of MEK (Alessi et al., 1995), on LysRS nuclear translocation in order to determine whether this translocation is affected by MAPK activity. As shown in Figure 4C, both of these inhibitors prevented the nuclear translocation of LysRS in immunologically activated mast cells. Thus, LysRS is translocated to the nucleus in a MAPK-dependent fashion following IgE and antigen stimulation.

In order to determine whether the production of Ap_4A is dependent on the MAPK pathway, RBL cells were treated with U0126, and Ap_4A production was examined. As seen in Figure 4D, MEK inhibition resulted in a significant decrease in Ap_4A levels in RBL cells. Thus, we concluded that the MAPK pathway is involved in the synthesis of Ap_4A by LysRS.

Subsequent to our observations of MAPK-dependent nuclear presence of LysRS and MAPK-dependent Ap_4A production, we wanted to determine if this nuclear localization is a prerequisite, exclusive condition for Ap_4A synthesis. To address this, we transfected cells with a LysRS S207A variant that was linked to a strong NLS. This variant was expressed in the nucleus despite the fact that it is not phosphorylated by ERK. There was no significant difference in Ap_4A production between the cytosolic and nuclear LysRS S207A expression (Figure S5B). Hence, nuclear localization by itself of the LysRS S207A variant cannot lead to induction of Ap_4A synthesis.

The Involvement of the MAPK Pathway in MITF Transcriptional Activity

To determine the direct effect of LysRS on the transcriptional activity of MITF, the transcript levels of two of its responsive genes, *tryptophan hydroxylase* (*TPH*) and *mast-cell protease 5* (*MCP5*) (Ito et al., 1998; Morii et al., 1997), were measured in immunologically activated RBL cells in which LysRS had been knocked down using the corresponding siRNA. These two target genes showed a marked decrease in their transcript levels in LysRS knockdown cells (Figures 5A and 5B), indicating that LysRS is significantly involved in the regulation of MITF transcriptional activity.

Culturing RBL cells with MAPK inhibitor prior to immunological activation prevented the process of LysRS dissociation from the MSC, phosphorylation, and translocation into the nucleus. The downstream effect of LysRS being retained in the cytoplasm was further examined by measuring Ap_4A levels and assessing MITF transcriptional activity by quantifying transcription of its target genes. The immunological induction of *TPH* and *MCP5* genes was completely blocked by treatment with U0126 (Figures 5C and 5D). Furthermore, the LysRS S207D variant was shown to enhance transcription of TPH compared to WT (Figure 5E). These results strongly indicate that in activated mast cells, LysRS is involved in the regulation of MITF transcriptional activity via Ser207 phosphorylation in the MAPK pathway.

DISCUSSION

Among the aaRSs family, LysRS is the major contributor to the production of Ap_4A (Wahab and Yang, 1985). LysRS is usually found in mammalian cells as a part of the MSC (Han et al., 2003; Robinson et al., 2000); however, in eukaryotic cells, it is several-fold more efficient at producing Ap_4A when found dissociated from the MSC (Wahab and Yang, 1985). Additionally,

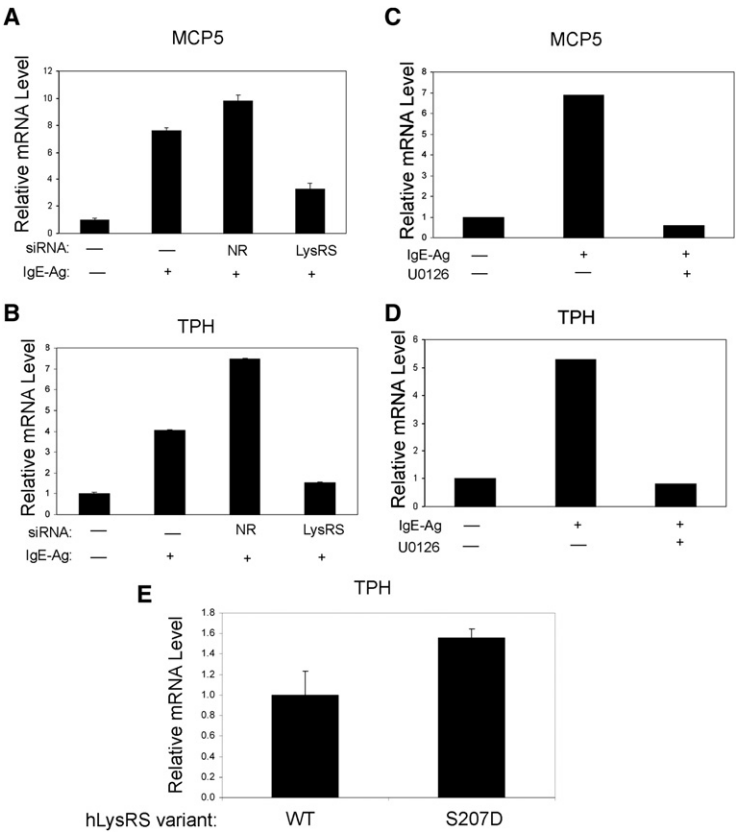

Figure 5. Determination of the Expression Levels of MITF-Responsive Genes in Cells Administered siRNA against LysRS

(A and B) The mRNA quantitation of MCP5 and TPH was determined by SYBR-green incorporation to real-time PCR in RBL cells. Expression levels were normalized to β-actin housekeeping gene. The mean and standard error of the mean (SEM) of three experiments is shown.

(C and D) RBL cells were activated for 4 hr by IgE-Ag with or without U0126. The mRNA quantitation of MCP-5 and TPH was determined by real-time PCR as described above. One of two independent experiments is shown.

(E) RBL cells were transfected with either WT or S207D hLysRS variants and activated for 4 hr with IgE-Ag. TPH mRNA level was determined by real-time PCR as described above. The mean and standard error of the mean (SEM) of five experiments is shown.

in vitro studies have shown that phosphorylation of aaRS enhances Ap_4A production (Dang and Traugh, 1989).

Using the siRNA technique, we have demonstrated directly that in mast cells, inhibition of LysRS expression in vivo caused a substantial decrease in the induction of Ap_4A in response to FcεRI aggregation. Our previous studies suggested that an increase in Ap_4A can induce the release of Hint-1 from MITF (Lee et al., 2004). Since the level of Ap_4A needed for this release was relatively high, and given that we have demonstrated the binding of LysRS to this transcription factor, we investigated by which pathway(s) LysRS could produce high amounts of Ap_4A leading to the release of Hint-1 from its bound transcription factor. Our assumption was that LysRS plays a pivotal role in Ap_4A production, and thus, we expected that this activity of LysRS would be regulated by an inducible process and would involve dissociation from the MSC.

The MSC, which contains three scaffold proteins and nine aaRSs, has been referred to recently as a "signalosome" (Han et al., 2006), since many of its aaRSs elements, aside from their catalytic roles in protein synthesis, are also involved in signaling pathways. Another aaRS with a "moonlighting," nonconventional role, GluProRS, has been shown to be regulated through its release from the MSC following interferon γ induction (Sampath et al., 2004). Similarly, the small form of arginine tRNA synthetase, which is located in the cytoplasm outside the MSC, was shown to play a role in posttranslational modification, whereas the longer form, which is responsible for translation, was found in the MSC (Ferber and Ciechanover, 1987).

Our results demonstrated that LysRS is released from the MSC following FcεRI aggregation of mast cells; this release is due to phosphorylation on a specific serine residue and is dependent on the induction of the MAPK pathway.

The MAPK pathway is one of the most important mast-cell pathways involved in signaling via the stimulus of FcεRI aggregation (Furuno et al., 2001; Santini and Beaven, 1993; Tsai et al., 1993). Moreover, MEK activation is required for antigen-stimulated secretion in mast cells (Hirasawa et al., 1995).

Our immunohistochemical studies and cellular fractionation assays revealed that a much greater proportion of LysRS can be found within the nucleus following FcεRI aggregation. Using MEK inhibitors, we have clearly demonstrated that this nuclear translocation was dependent upon activation of the MAPK pathway. Interestingly, both reduction of cellular levels of LysRS by specific siRNA and the use of the MEK inhibitor U0126 led to reduced mRNA levels of several target genes of MITF in mast cells.

It has previously been reported that the MAPK cascade is critical in the activation of various transcription factors, such as ATF3 and AP-1, and it is also involved in cytokine production in mast cells (Garrington et al., 2000). Here we have revealed for the first time a new MAPK pathway branch in activated mast cells that leads to gene expression via the phosphorylation of LysRS and the network of MITF and Hint-1.

Combining all of our data, we propose a model in which LysRS is phosphorylated on the serine 207 residue through the MAPK pathway following cellular activation. This phosphorylation is followed by the release from the MSC of LysRS, which then translocates into the nucleus. The released serine 207 phosphorylated LysRS can then produce higher levels of Ap_4A, with profound cellular effects via binding to Ap_4A binding proteins. One such effect is the removal of the repressor Hint-1 from MITF, enabling it to transcribe its target genes.

Direct binding of LysRS to molecules such as transcription factors should allow very high levels of Ap_4A in the vicinity of these molecules (Figure 6). Thus, based on our data, we propose that in immunologically activated cells, LysRS has a signal transduction role besides its other well-defined roles.

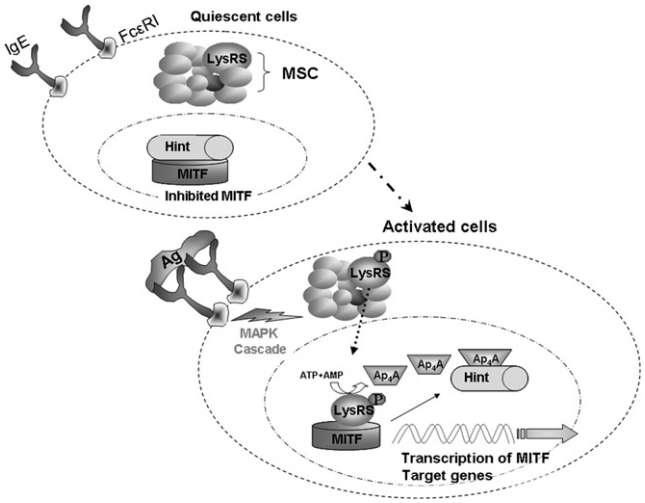

Figure 6. Proposed Model for LysRS as a Signaling Molecule
Following specific stimuli, LysRS is serine phosphorylated in a MAPK-dependent fashion, dissociates from the MSC, and translocates from the cytoplasm to the nucleus. The phosphorylation on serine residue 207 elevates Ap_4A levels, leads to the dissociation of Hint-1 from MITF, and allows this transcription factor to activate its responsive genes.

EXPERIMENTAL PROCEDURES

Antibodies
The antibody anti-LysRS was custom made against a specially designed determinant KEVLLFPAMKPE (Hy Laboratories Ltd., Israel). Anti-phosphoserine antibody was purchased from Zymed Laboratories (San Francisco, CA). Anti-phosphothreonine and anti-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MetRS and anti-ArgRS were purchased from Abcam (Abcam Ltd., Cambridge, UK). Anti-p43/AIMP1 was produced as previously described (Han et al., 2006).

Ap_4A Determination
This assay detects the relative amount of Ap_4A present in extracts of mammalian cells. For each determination, cells were grown to about 80% confluence. The cell layer was lysed with trichloroacetic acid. Extraction and measurement by luminometry of the nucleotides were performed as described previously (Murphy et al., 2000). Results were normalized by Bradford protein assay.

Cell Growth
RBL-2H3 cells were maintained in RPMI 1640 medium as previously described (Razin et al., 1999). RBL cells were sensitized first with anti-DNP IgE monoclonal antibody (SPE-7, Sigma-Aldrich Corp., St. Louis, MO) and then challenged with DNP (Sigma-Aldrich Corp.). IgE antibody was centrifuged (18,000 g, 5 min) before use to remove aggregates.

Chemical Inhibitor Treatment
U0126, PD098059, and SB203580 were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Plasmid Construction
Human LysRS was subcloned into the EcoRI and *Xba*I sites of the pSC2+MT vector (Invitrogen). This vector was used for the production LysRS mutant by site-directed mutagenesis in which serine was replaced by alanine at both 207 and 470 positions (LysRS S207A/S470A). Human LysRS S207A variant was subcloned into pCMV/myc/cyto and pCMV/myc/nuc vectors. The fidelity of all constructs was verified by direct sequencing.

Gel Filtration Chromatography of Cell Lysates
Cell extracts were applied to a Superdex 200 column (30 × 1 cm from Amersham Biosciences) using AKTA Explorer (Amersham Biosciences) and eluted at a flow rate of 0.8 ml/min in buffer containing 20 mM Tris-HCL (pH 7.4), 150 mM NaCl, 10% glycerol, and 0.5% Triton X-100. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (163 kDa), BSA (67 kDa), and OvaAlb (44 kDa) were used as molecular weight standards. The eluted proteins in each fraction were analyzed by immunoblotting with anti-LysRS.

Gel Electrophoresis and Western Blots
Proteins were resolved by 10% SDS-PAGE under reducing conditions and were transferred to nitrocellulose membranes. Visualization of reactive proteins was performed by enhanced chemiluminescence.

Immunoprecipitation
The immunoprecipitation of the specific proteins from RBL cells was carried out as previously described (Levy et al., 2002).

Two-Dimensional Electrophoresis
2D electrophoresis was performed as previously described (Han et al., 2008). Cells were solubilized in 2-D-lysis buffer (7 M urea, 2 M thiourea, 4% w/v, CHAPS, 100 mM DTT). Cell lysates were loaded to immobilized pH gradient strip gels (linear pH gradient 4–7, 7 cm). Isoelectric focusing was performed at 4,000 V until the total volt-hours reached 10 kV hours using PROTEAN IEF cell (Bio-Rad). Following two-step equilibration with 375 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, 2% DTT, and 2.5% iodoacetamide, the IPG strips were embedded on top of 8% SDS-PAGE gels and sealed with 2% agarose. Proteins were separated based on their molecular weight.

Expression and Purification of LysRS Proteins
cDNA of human LysRS WT or S207A were subcloned into pET28a (Novagen). Recombinant LysRS proteins were expressed as His fusion proteins and purified by Ni^{2+}-bound His-Bind resin. Cells were lysed and sonicated in 30 ml of lysis buffer (20 mM KH2PO4, 500 mM NaCl, 2 mM β-mercaptoethanol [pH 7.8] containing 0.5 mM PMSF, 1 μg/ml leupeptin, and 5 μg/ml aprotinin). The lysates were centrifuged at 20,000 g for 1 hr at 4°C. The supernatant was incubated with 1 ml of Ni^{2+}-bound His-Bind resin at 4°C overnight with constant agitation. The resin was washed with 10 column volumes of washing buffer A (20 mM KH2PO4, 500 mM NaCl, 2 mM β-mercaptoethanol, and 10% glycerol [pH 6.0]), washing buffer B (20 mM KH2PO4, 500 mM NaCl, 2 mM β-mercaptoethanol, and 10% glycerol [pH 5.2]), and washing buffer C (20 mM KH2PO4, 500 mM NaCl, 2 mM β-mercaptoethanol [pH 7.8], and 50 mM imidazole). Bound protein was eluted with elution buffer (20 mM KH2PO4, 500 mM NaCl, and 2 mM β-mercaptoethanol [pH 7.8]) containing 300 mM imidazole. The fractions eluted with 300 mM imidazole buffer were pooled and injected onto PD-10 gel-filtration columns equilibrated with phosphate-buffered saline. The fractions were then analyzed by SDS-PAGE and western blotting.

In Vitro Phosphorylation of LysRS by ERK1
LysRS proteins (200 ng) were incubated with 20 ng of recombinant ERK1 in phosphorylation buffer (20 mM Tris/HCl [pH 7.5], 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT, 0.12 mM ATP, and 2 μCi [γ-32P] ATP [3000 Ci/mmol]) for 15 min. The reaction mixture was then electrophoresed through an 8% SDS-polyacrylamide gel. The dried gel was exposed to autoradiography.

Real-Time Quantitative Polymerase Chain Reaction
Candidate MITF responsive gene transcription was measured using real-time quantitative PCR. mRNAs of MITF target genes were quantified by SYBR-green incorporation (ABgene SYBR green ROX Mix, ABgene). Real-time PCR was performed on Rotor-Gene sequence detection system (Corbett, Australia). The genes whose mRNA levels were quantified by real-time PCR were rat *TPH*, *MCP5*, and *β-actin*.

siRNA

Cells were transfected with a siRNA duplex consisting of two complementary 21 nucleotide RNA strands with 3′ dTdT overhangs (QIAGEN Inc., CA) in order to downregulate LysRS. siRNAs were designed to be complementary to nucleotide sequence found in rat mRNA of LysRS, and a nonrelevant nucleotide sequence was used as the control (NR siRNA). The target sequence of the specific siRNAs for LysRS was TTCGTTCACATCAATAACAAA. The nonrelevant control sequence was AATTCTCCGAACGTGTCACGT.

Transfection

Amaxa Nucleofector technology (Amaxa, Cologne, Germany) was used for transfecting cells. 2 × 10^6 cells were transfected with 3 μg of the selected siRNA oligonucleotide according to the manufacturer's protocol. Briefly, the cells were resuspended in 100 μl nucleofector solution. RNA was added, and the mixture transferred into an electroporation cuvette. Nucleofector solution was used to stabilize the cells during electroporation, which was performed using the T-20 program. The cells were suspended in 2 ml of cell culture medium immediately after electroporation.

Indirect Fluorescent Immunocytochemistry

RBL cells were grown on glass coverslips in 6-well plates. After extensive washing with PBS, the cells were fixed with 1.5 ml 4% formaldehyde in PBS for 10 min. The fixed cells were then washed with PBS and permeabilized with 1.5 ml Triton 100× diluted 1:2 with PBS containing 7.5 mg bovine serum albumin. After 45 min blocking with normal donkey serum, the cells were stained either with rabbit anti-LysRS followed by the addition of Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA). Fluorescence analysis was performed using Zeiss LSM 410 confocal laser scanning system connected to Zeiss Axiovert 135M microscope (Zeiss, Germany) as previously described (Sonnenblick et al., 2004).

SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00352-9.

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