RNase L dimerization in a mammalian two-hybrid system in response to 2′,5′-oligoadenylates

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ABSTRACT

RNase L, a key enzyme in the anti-viral activity of interferons, requires activation by 2′,5′-linked oligoadenylates (2–5A) to cleave viral and cellular single-stranded RNA. Here we demonstrate that 2–5A causes formation of stable dimers of RNase L in intact human cells as measured with a mammalian two-hybrid system. Hybrid proteins consisting of the GAL4 DNA binding domain fused to RNase L and the VP16 transactivation domain fused to RNase L were able to associate and drive transcription of a reporter gene, but only after cells were transfected with 2–5A. Several functional forms of 2–5A, such as p3A2′p5′A2′p5′A, were capable of activating transcription in human HeLa cells. In contrast, p3A2′p5′A, which can neither activate nor dimerize RNase L, did not induce gene expression. Evidence for the involvement of the C-terminal region of RNase L in dimerization was obtained by expressing truncated forms of RNase L. These findings describe a convenient, high-throughput screening method for RNase L activators which could lead to the discovery of novel anti-viral and anti-cancer agents.

INTRODUCTION

Interferons provide a primary line of defense against virus infections by inducing genes which encode anti-viral proteins, including the 2′,5′-linked oligoadenylates (2–5A)-synthetases (1). These synthetases require double-stranded RNA, generated by the infecting virus, to produce a series of short 2′,5′-linked oligoadenylates (2–5A) from ATP (2). Accordingly, 2–5A has been observed in interferon-treated mammalian cells infected with encephalomyocarditis virus (EMCV), vaccinia virus, reovirus, herpes simplex and SV40 (3). The function of 2–5A is to activate a latent endoribonuclease, the 2–5A-dependent RNase L, resulting in the rapid decay of viral and cellular single-stranded RNA (4). The physiological role of the 2–5A system was investigated in RNase L-null mice which are defective for the anti-EMCV effect of interferon and show apoptotic defects in the thymus gland (5).

RNase L has an interesting arrangement of structural and functional domains. An isolated N-terminal half, containing nine ankyrin-repeats and two P-loop motifs, binds 2–5A but lacks RNase activity. An isolated C-terminal half cleaves RNA in the absence of 2–5A (6). Therefore, the regulatory and a catalytic functions of RNase L are present in the N- and C-terminal halves, respectively. Previous studies involving biochemical and biophysical methods demonstrated that purified, recombinant RNase L, dimerized during the 2–5A-dependent activation process (6–8). Therefore, dimerization is intimately-linked to functional activation of the ribonuclease. However, the significance of these observations has remained obscure in the absence of in vivo evidence for the self-association of RNase L. In this regard, protein–protein interactions in cell-free systems do not necessarily reflect the situation in intact living cells.

To monitor and study RNase L dimerization in response to 2–5A, we have adapted the two-hybrid system originally developed for yeast by Fields and Song (9). Two-hybrid systems utilize fusion proteins to detect protein–protein interactions via the activation of reporter gene expression. They are based on the observations that many eukaryotic transcriptional activators (including GAL4) consist of two physically separable modular domains; one acting as a DNA-binding domain the other as a transcriptional activation domain (10). For instance, a mammalian two-hybrid system uses the transactivation domain of the herpes simplex virus VP16 protein and the GAL4 DNA-binding domain (11–13). Two-hybrid assays provide sensitive methods for detecting weak or transient interactions, probably the norm in many physiological complexes. Because the assays are performed in intact mammalian cells, the proteins are likely to be properly modified and in their native conformations. Two-hybrid systems have been used to identify novel proteins (from a library of proteins) that interact with a protein of interest, test known proteins for interaction, define protein domains or specific amino acid residues required for interaction between two known proteins and identify compounds that affect these interactions (reviewed in 14).

Here we have developed a mammalian two-hybrid system in which chimeras of RNase L control the expression of a reporter gene in response to 2–5A. These findings provide the first evidence that 2–5A induces dimerization of RNase L in intact cells. This method will allow mapping of the RNase L dimerization domain and screening for RNase L activators that could function as anti-viral or anti-cancer drugs.

MATERIALS AND METHODS

Construction of plasmids

Maps of the plasmids used in the study are shown (Fig. 1). Plasmid pSG424 (encoding the GAL4 DNA-binding domain),

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Figure 1. Plasmid maps for the RNase L/two-hybrid system. Poly A, the SV40 polyadenylation signal sequence; TA, transactivation sequence; BD, DNA binding domain.

Assay for expression of the fusion proteins in HeLa cells

To demonstrate the expression of the Gal4 binding domain fused to RNase L (Gal4BD-RNase L) and the VP16 transactivating domain fused to RNase L (VP16TA-RNase L), transient transfections were done in HeLa M cells. HeLa cells were plated to 75% confluency the day before the transection in 10 cm Petri dishes. Cells were washed with PBS and fresh DMEM containing 10% FBS was added −1 h prior to transfection. Transfections of plasmid DNAs (8 µg) were done using lipofectamine PLUS transfection reagent according to the manufacturer's protocol ( Gibco-BRL ). Just before the transfections, cells were washed with PBS, and then with OptiMEM. Transfections were done in the presence of OptiMEM. Cells were incubated with the DNA for 6–7 h and were then washed with PBS and incubated overnight with DMEM containing 10% FBS. Cells were split into 150 mm Petri dishes and grown an additional 24 h. Protein extracts were made in NP-40 lysis buffer (0.5% v/v NP-40, 90 mM potassium chloride, 1 mM magnesium acetate, 10 mM HEPES, pH 7.6 and 2 mM mercaptoethanol) containing the protease inhibitor, leupeptin (100 µg/ml) (18). Cell extracts, containing 150 µg of protein per sample, were denatured and electrophoresed on 10% SDS–polyacrylamide gels. Proteins were transferred to Immobilon-p membranes (Millipore) using a Tris–glycine–methanol buffer. Fusion proteins, as well as endogenous RNase L, were detected on the blots using monoclonal antibody against human RNase L (22) for 1 h at room temperature followed by 4°C for 16 h. Actin was detected with a monoclonal antibody to a highly conserved region of actin (clone C4; Boehringer-Mannheim) (1) for 1 h at room temperature (added after the incubations with the antibody against RNase L). Detection was done using anti-mouse IgG/ horseradish peroxidase (Gibco-BRL) (1) and the ECL detection system (Amersham).

2′,5′-Oligoadenylates

The p3A(2′p5′A)2 was synthesized from ATP with HeLa cell 2′–5A-synthetase and purified as described (18). The pA(2′p5′A)3, SpA(2′p5′A)3 and SpA(2′p5′A)3-anti-PKR3′-3′C [subsequently 'SpA(2′p5′A)3-antisense'] (19) were chemically synthesized and kindly provided by Guying Li (Cleveland). The p3A2′p5′A and pA2′p5′A3 were generous gifts from Paul F. Torrence (Bethesda).

Transfections and assays for luciferase and β-galactosidase activities

HeLa cells were cultured in 6-well dishes to 80% confluency in Dulbecco's modified medium (DMEM) plus 10% fetal bovine serum (FBS). Plasmids (1 µg each) in 100 µl of OPTIMEM (Gibco-BRL) were mixed with 6 µl of lipofectamine (Gibco-BRL) in 100 µl of OPTIMEM and incubated at room temperature for 20 min. Cell culture media was aspirated, cells were washed twice and 0.8 µl of OPTIMEM was added to each well. The DNA/lipofectamine mixture was added dropwise to the cells and incubated for 6 h at 37°C. Cells were washed twice with PBS and incubated with DMEM plus 10% FBS for 15 h. Cells were then transfected with 2–5A using reagents in an MBS/mammalian transfection kit (Stratagen). A calcium phosphate precipitate of 2–5A was prepared in HEPES-buffered saline, pH 7.05, plus 125 mM of CaCl2. After aspirating the media, washing cells twice with PBS, and adding 1.5 µl of DMEM supplemented with...
either 6% MBS or 10% FBS, the precipitates were pipetted onto cells and incubated at 37°C for 90 min. The mixtures were aspirated and the monolayers were incubated with DMEM containing 10% FBS for ~22 h. Cells were harvested by scraping in PBS, centrifuged for a few seconds in a microfuge, resuspended in 60 µl of reporter lysis buffer (Promega), incubated at room temperature for 15 min, vortexed briefly, freeze/thawed once on dry ice/37°C, centrifuged for 5 min at 10000 g, and the supernatants were collected and assayed for luciferase activity as described (Promega). Alternatively, 2–5A were incubated with 30 mg/ml of DEAE-dextran at room temperature for 10–15 min (20). The mixtures were diluted in OPTI MEM in 100 µl, and added to cells from which media was removed. OPTI MEM, 0.9 ml, was immediately added and cells were incubated for 90 min at 37°C. Cells were incubated for 22 h with fresh media and extracts were prepared as described above.

To determine the transfection efficiency, β-galactosidase activity was measured by determining the optical density at 420 nm after incubation at 37°C of cell extracts with o-nitrophenyl-β-D-galactopyranoside (Sigma) (20). Results are expressed as the ratio of the luciferase activity/β-galactosidase activity, normalized so that the ratio in the control cells equals 1.0. Control cells were transfected with pGal/hRL, pVP16/hRL, pGal5/luc and pCMV/β-gal in the absence of 2–5A.

RESULTS AND DISCUSSION

Design of plasmids for the two-hybrid system and expression in HeLa cells

To establish whether RNase L forms dimers in intact cells, and to create a convenient, high-throughput method for measuring a biological response to 2–5A, hybrids of RNase L were generated (Figs 1 and 2). Plasmids pGAL/hRL and pVP16/hRL encode the yeast GAL4 DNA-binding domain and the HSV VP16 transactivation domain, respectively, fused to the N-terminus of human RNase L. N-terminal fusions of RNase L were made, instead of C-terminal fusions, because glutathione-S-transferase fusions to the N-termini of RNase L are fully functional (6,21).

Previously, we implicated a C-terminal region of RNase L as being important for dimer formation in cell-free systems (6). Therefore, to determine if the C-terminal tail of RNase L is required for dimer formation in intact cells, hybrid proteins lacking 80 C-terminal amino acids of RNase L were constructed (see plasmids pGal/hRLΔ80 and pVP16/hRLΔ80, Fig. 1). To obtain mechanistic information about a dominant negative mutant of the murine RNase L, lacking a C-terminal tail, RNase LΔZBI was fused to the VP16 transactivation domain (Fig. 1, plasmid pVP16/mRL-ZBI) (17). The reporter plasmid, pGal5/luc, contains five Gal4 binding sites upstream of a minimal E1B promoter and the luciferase coding sequence. The plasmid, pCMV/β-gal, containing a CMV promoter linked to β-galactosidase cDNA, was used to control for transfection efficiency. Dimerization of RNase L was indirectly measured by the luciferase activity normalized for β-galactosidase activity.

To demonstrate that the fusion protein could be expressed in cells, plasmids pGal/hRL and pVP16/hRL were individually transfected into HeLa cells (Fig. 2). Western blots prepared from extracts of the transfected cells were probed with monoclonal antibodies against human RNase L and actin. In the control, non-transfected cells, the endogenous RNase L (83.5 kDa) and actin isoforms (42 kDa) were clearly seen (lane 1). In cells transfected with pGal/hRL, the GAL4BD-RNase L fusion protein was also observed near its expected position (the GAL4 BD is 17 kDa) (lane 2). In addition, there was an apparent breakdown product of the GAL4BD-RNase L just below the intact fusion protein (second band from top in lane 2). Cells transfected with pVP16/hRL produced the VP16TA-RNase L fusion protein which migrated to a position relative to the molecular weight markers which is slightly larger than its actual size of 92 kDa (lane 3).

A comparison of the relative efficiencies of different methods for introducing 2–5A into HeLa cells

To determine if the RNase L fusion proteins could interact in cells, a mixture of plasmids pGal/hRL, pVP16/hRL, pGal5/luc and pCMV/β-gal was transfected into HeLa cells using lipofectamine. After 15 h, the cells were transfected with 1 µM of p5A(2′p5′A)2 by calcium phosphate co-precipitation in the presence of FBS. Luciferase and β-galactosidase activities were determined after an additional 22 h of cell culture. A 2.7-fold increase in the ratio of luciferase to β-galactosidase demonstrated that dimerization of the hybrid proteins occurred in the cells (Fig. 3). However, calcium-phosphate co-precipitation in media containing FBS caused the death of a substantial proportion of the cells when used in conjunction with lipofectamine. DEAEX-dextran was even less effective than the calcium-phosphate/FBS technique, resulting in a 2.2-fold relative increase in luciferase activity. The MBS method, which involves calcium-phosphate co-precipitation in the presence of a modified bovine serum, was the least harmful to the cells of the three methods, and resulted the highest ratio of luciferase to β-galactosidase activity, a 3.7 ± 0.6-fold increase (Fig. 3). Therefore, in all subsequent experiments the 2–5A were introduced into the cells by the MBS method.

Formation of RNase L dimers in human cells requires the presence of a 2–5A

To determine the effect of 2–5A on RNase L dimerization, cells were transfected with the mixture of pGal/hRL, pVP16/hRL,
Figure 3. Optimizing the two-hybrid system by comparing three methods of introducing p3A(2′p5′A)2 (1 µM) into HeLa cells. The control represents cells transfected with the plasmids without subsequent transfection with 2–5A. The y axis represents the ratio of the luciferase/β-gal activities relative to the arbitrary control level (1.0). Standard deviation bars are shown. The results were the average of two to four experiments each done in triplicate.

Figure 4. RNase L dimerization in HeLa cells as a function of p3A(2′p5′A)2 concentration. Results were the average of four assays with pGal/hRL, pVP16/hRL, pGal5/luc and pCMV/β-gal and are shown with standard error bars.

pGal5/luc and pCMV/β-gal, followed after 15 h with either a mock transfection, or transfections with different amounts of p3A(2′p5′A)2 (Fig. 4). In the absence of p3A(2′p5′A)2 there was only a very low basal activity of luciferase activity, suggesting the RNase L does not dimerize unless 2–5A is present. In contrast, a dose-dependent increase of luciferase/β-galactosidase was observed in cells transfected with p3A(2′p5′A)2. RNase L dimerization was clearly detected at ≤200 nM of p3A(2′p5′A)2. The maximal relative increase of luciferase activity, 4.0 ± 0.56-fold, was obtained with 2 µM of p3A(2′p5′A)2. These findings demonstrate that 2–5A induces dimers of RNase L in intact human cells.

Figure 5. Dimerization of RNase L requires functional 2–5A. Cells were transfected with pGal/hRL, pVP16/hRL, pGal5/luc and pCMV/β-gal. The 2–5A were present at 1 µM each. Results are an average of two to four experiments each done in duplicate, with standard deviation bars.

Only functionally active forms of 2–5A cause RNase L dimers to form in human cells

Activation of RNase L requires 2–5A that contain a minimum of three adenylyl residues (22, and references therein). To determine if the dimerization of RNase L was specific for functional forms of 2–5A, we compared a panel of 2–5A in the two-hybrid system (Fig. 5). The dimer, p3A2′p5′A, failed to induce luciferase activity. Similarly, in a previous study we showed that p3A2′p5′A fails to cause RNase L to dimerize in vitro (7). In contrast, p3A2′p5′A and p3A3′p5′A resulted in an ~4-fold increase in luciferase/β-galactosidase activity, while the monophosphate, pA(2′p5′A)3, and the thiophosphate, SpA(2′p5′A)3, caused an ~2-fold increase. All four of these compounds, p3A2′p5′A, p3A3′p5′A, pA(2′p5′A)3 and SpA(2′p5′A)3 activate RNase L in vitro to a similar extent (22-23). The reduced activity in the two-hybrid system of pA(2′p5′A)3 compared to 5′-triphosphorylated 2–5A may be due to less efficient uptake due to a less negative charge and a loss of the critical α-phosphoryl group by cellular phosphatases. On the other hand, the lower activity of SpA(2′p5′A)3, which is resistant to phosphatase, may be due only to less efficient uptake (23). In addition, a 2–5A antisense chimera containing SpA(2′p5′A)3 linked to antisense DNA, which can activate RNase L but at reduced levels compared to 2–5A per se, produced a 2.3-fold increase (24).

Mapping the dimerization domain of RNase L by the two-hybrid system

Previously, we showed with purified recombinant GST-fusion proteins that the C-terminal tail of RNase L is essential for ribonuclease activity and dimer formation, but not for 2–5A binding activity (6). To determine if the C-terminal tail of RNase L is required for dimer formation in cells, truncated hybrid
The C-terminal tail of RNase L is involved in the dimerization process. Luciferase activity was measured in the presence of 1 μM of pA(2′p5′A)3 (or pA(2′p5′A)3 in one of two experiments with pGal/mRL2B1). The different combinations of plasmids used (together with pGal5/luc and pCMV/β-gal) are indicated. The control contained pGal/hRL and pVP16/hRL, pGal5/luc and pCMV/β-gal in the absence of 2–5A. Results are averages of one to three experiments, each done in triplicate or quadruplicate, and are shown with standard deviation bars.

The two hybrid system is a method for monitoring RNase L dimerization in intact cells

The 2–5A pathway provides opportunities for the development of therapeutic agents because RNase L is present in most mammalian cell types and can be activated by a small molecule, 2–5A. Furthermore, because RNase L activation is implicated in the anti-viral and anti-cellular activities of interferons, novel activators of RNase L could have anti-viral or anti-cancer activities (5,17). The two-hybrid system is thus a convenient, high-throughput method for screening potential RNase L activators. The assay can also be used to monitor uptake of RNase L activators (Fig. 5) and it might detect the natural occurrence of 2–5A in intact cells under different physiological conditions, such as following interferon-treatment and virus-infection. It will also be possible to generate a map of the RNase L interaction domains by cloning additional mutant forms of RNase L in the two-hybrid system vectors (Fig. 6). Finally, the ability of RNase L to dimerize in response to a small molecule provides a novel method for the control of gene expression in vivo. This method is the second example of a two-hybrid system that depends on a small molecule for the interaction of the hybrid proteins. In the first case, dimerization of FKBP12 and a FRAP fragment was mediated by rapamycin thus controlling transcription in vivo (25). In the case of RNase L, it is unknown if the 2–5A unmask a protein–protein interaction domain which leads to dimer formation or if 2–5A bridges are formed between RNase L monomers. However, the observation that one molecule of 2–5A binds to each monomeric RNase L molecule favors the former hypothesis (8).

Because 2–5A can both activate and dimerize RNase L, the extent of luciferase activity is limited by RNA degradation. Inhibitory effects of 2–5A transfections on protein and RNA synthesis are transient as a result of the rapid decay of 2–5A in cells (26). It is apparent from our results that at least some of luciferase mRNA escapes to the cytoplasm and is translated into functional protein. However, as a future development of the method, it would be useful to disable the RNase domain by mutation without affecting the dimerization activity. Alternatively, perhaps a small molecule can be found or chemically-synthesized which can enter cells and dimerize RNase L without causing its catalytic activation. Hence, this assay will provide a convenient method for screening RNase L activators and in the testing and design of small molecule modulators of RNase L.

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