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The La antigen inhibits the activation of the interferon-inducible protein kinase PKR by sequestering and unwinding double-stranded RNA

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ABSTRACT

The La (SS-B) autoimmune antigen is an RNA-binding protein that is present in both nucleus and cytoplasm of eukaryotic cells. The spectrum of RNAs that interact with the La antigen includes species which also bind to the interferon-inducible protein kinase PKR. We have investigated whether the La antigen can regulate the activity of PKR and have observed that both the autophosphorylation of the protein kinase that accompanies its activation by dsRNA and the dsRNA-dependent phosphorylation of the α subunit of polypeptide chain initiation factor eIF-2 by PKR are inhibited in the presence of recombinant La antigen. This inhibition is partially relieved at higher concentrations of dsRNA. Once activated by dsRNA the protein kinase activity of PKR is insensitive to the La antigen. We have demonstrated by a filter binding assay that La is a dsRNA binding protein. Furthermore, when recombinant La is incubated with a 900 bp synthetic dsRNA or with naturally occurring reovirus dsRNA it converts these substrates to single-stranded forms. We conclude that the La antigen inhibits the dsRNA-dependent activation of PKR by binding and unwinding dsRNA and that it may therefore play a role in the regulation of this protein kinase in interferon-treated or virus-infected cells.

INTRODUCTION

The La antigen is a 46.7 kDa cellular protein, located in both nucleus and cytoplasm, that interacts with a variety of RNA molecules. RNA species that bind the La antigen include the small cellular Y RNAs, which are constituents of the cytoplasmic Ro RNP particles (1, 2), and virally encoded species such as adenovirus VA_I RNA (3) and Epstein–Barr virus EBER-1 and EBER-2 (4). Bachmann *et al.* (5) have reported that the La antigen has a DNA–RNA unwinding activity and recent evidence from the same group has suggested that this protein also has dsRNA unwinding activity against a variety of substrates (M.Bachmann, personal communication). The physiological function of La remains unknown, although it has been suggested that it may play a role in the termination of transcription by RNA polymerase III (6, 7) or in RNA transport from nucleus to cytoplasm (8, 9). Recently evidence for a role in the regulation of translation of poliovirus RNA has been reported (10). Antibodies against the La antigen are commonly found in sera from patients suffering from autoimmune disorders such as systemic lupus erythematosus and Sjögren's syndrome.

Amongst the molecules with which the La antigen can interact are a number of RNA ligands that also bind to and regulate the activation of the interferon (IFN)-inducible protein kinase PKR (previously called DAI or p68) (4, 11–14). Examples of such RNAs include VA_I RNA and the EBERs, described above. PKR is a 62 kDa enzyme that is present at low levels in most mammalian cells and can be induced up to 10-fold by interferon treatment. It is present in both the cytoplasm as a ribosome-associated protein and in the nucleus where it is specifically located in the nucleolus (15, 16). This enzyme has been the subject of intensive study in recent years because of its likely involvement in the antiviral and antiproliferative effects of the interferons (17). PKR binds double-stranded RNA (dsRNA) with high affinity and is dependent on low concentrations of dsRNA for its activation, a process which is accompanied by autophosphorylation of the enzyme. When activated by viral dsRNA molecules in infected cells PKR phosphorylates its principal known substrate, the α subunit of the eukaryotic protein synthesis initiation factor eIF-2 (18–21). Phosphorylation of eIF-2α contributes to the development of an antiviral state by inhibiting the activity of the guanine nucleotide exchange factor eIF-2B and thus impairing the overall rate of protein synthesis in infected cells (18, 22). Recently a potential role for PKR in cell growth regulation has also been suggested by the observations that over-expression of the human enzyme in yeast cells inhibits cell proliferation (23) and that high level expression of catalytically inactive mutant forms of the kinase in 3T3 cells

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converts the cells to a tumourigenic phenotype (24, 25). It has been suggested that the PKR mutants exert a dominant negative effect on the activity of the wild-type enzyme.

Many viruses have evolved mechanisms for blocking the activation of PKR (26). Well studied examples of such strategies include the production of large quantities of small viral RNA species that bind to the protein kinase and inhibit the dsRNA-mediated activation process (11), and the synthesis of viral proteins that sequester dsRNA activators (27–29). The dominant negative effect of at least one mutant form of PKR on the activity of the wild-type enzyme has also been shown to be due to competition between the two proteins for binding of dsRNA (30).

Although it is not yet clear what factors might regulate the activity of PKR in uninfected cells, other proteins that bind dsRNA may be involved. In view of the similar nature of the RNA ligands that interact with PKR and the La antigen, and the proposed dsRNA unwinding ability of the latter, we have investigated whether La can play a role in the control of the activation of PKR by dsRNA. We present evidence that recombinant La antigen inhibits the activation but not the activity of PKR and that the molecular basis of this effect is related to the ability of La to bind and unwind dsRNA.

MATERIALS AND METHODS

Materials

Rabbit liver PKR was purified and used as described previously (14). Recombinant human La antigen was expressed in *E.coli* strain BL21(DE3)pLysS as described by Slobbe *et al.* (1). The protein was subsequently purified from the bacterial lysate by ion exchange column chromatography using DEAE–cellulose (Whatman DE52) and phosphocellulose (Whatman P11) resins. DEAE–cellulose chromatography was performed with a buffer containing 25 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA and 1 mM dithioerythritol. The flow-through fraction from this column, containing the La antigen, was applied to a phosphocellulose column which was equlibrated with 50 mM Hepes–KOH (pH 7.9), 100 mM NaCl, 1 mM EDTA and 1 mM dithioerythritol. Bound material was eluted with a linear gradient of NaCl; the La antigen eluted at approximately 600 mM NaCl. The synthetic peptides NIEGMILLSELSRRRIRSIN (peptide S51) and NIEGMILLSELARRRIRSIN (peptide A51) were a gift from Dr S.Goodbourn (St George's Hospital Medical School, London). Reovirus double-stranded genomic RNA and rabbit reticulocyte eIF-2 were kindly provided by Dr D.H.Levin (Massachusetts Institute of Technology, USA). The synthetic dsRNA poly(I).poly(C) was purchased from Sigma Chemical Company. ^{32}P-labelled nucleotides and radioactive protein molecular mass markers were from New England Nuclear or Amersham International.

Protein phosphorylation assays

Autophosphorylation of PKR and the phosphorylation of eIF-2 by purified PKR were assayed essentially as described previously (13, 31). The incubation conditions were: 10 mM Tris–HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 5 μM EDTA, 47 μM NaF, 10% (v/v) glycerol, 10 μM ATP and 2.5 μCi [γ-^{32}P]ATP in a final reaction volume of 15 μl. After 15 min at 30°C the proteins were denatured with SDS gel sample buffer and subjected to electrophoresis on 15% SDS gels. The dried gels were analysed by autoradiography.

Peptide phosphorylation assay

Phosphorylation of the synthetic peptides S51 and A51 (see above) was determined under the conditions described previously (14). Radioactivity in peptides bound to P81 phosphocellulose paper (Whatman) was measured by liquid scintillation counting.

Synthesis of labelled dsRNAs

Three different dsRNA substrates were used to analyse the binding and unwinding characteristics of the La antigen. A 940 bp dsRNA was prepared by *in vitro* symmetrical transcription of exon 2 of the mouse c-*myc* gene using T7 and SP6 RNA polymerases for synthesis of the two respective strands (12). The strand transcribed by T7 polymerase was radioactively labelled by inclusion of [α-^{32}P]UTP (5 mCi/ml) in the transcription mix. The two strands were synthesized separately, phenol extracted, heated and annealed to produce the radioactive dsRNA, which was then purified by CF11–cellulose chromatography as described previously (12). Poly(I).poly(C) and reovirus genomic dsRNAs were 3′ end-labelled using T4 RNA ligase and ^{32}P-pCp (14). The RNAs were separated from the unincorporated labelled nucleotide by Sephadex G-100 chromatography and then phenol extracted.

Analysis of La–RNA interaction by filter binding

The ability of purified recombinant La antigen to bind dsRNA was assessed by a filter binding assay (14). Increasing concentrations of the protein were incubated in 25 μl reaction volumes with ^{32}P end-labelled poly(I).poly(C) (2×10^6 counts per min/ml), in the presence of 10 mM Tris–HCl (pH 7.5), 80 mM KCl, 20 mM NaCl, 0.8 mM Mg acetate and 200 μg/ml bovine serum albumin. After 15 min at 30°C the incubations were diluted with 75 μl of wash buffer (containing the same ionic components) and rapidly passed through Millititer cellulose nitrate filters (Millipore) as described previously (14). After four washes with the same buffer the filters were cut out and dried and the bound radioactivity determined by scintillation counting.

Analysis of RNA binding and unwinding by gel electrophoresis

The 940 bp double-stranded c-myc exon 2 transcript, labelled with ^{32}P on one strand, was incubated with various concentrations of the La antigen in the presence of 20 mM Tris–HCl (pH 7.5) and 80 mM KCl, without added ATP, for 15 min at 30°C. The complexes were analysed by electrophoresis on non-denaturing gels.

End-labelled reovirus genomic dsRNA was incubated with La (25 μg/ml) for up to 60 min at 30°C in a 25 μl reaction volume. In this case the ionic conditions described by Bass and Weintraub (32) were used (25 mM Tris–HCl, pH 7.9, 25 mM KCl, 75 mM NaCl, 50 μM EDTA, 0.25 mM dithiothreitol, 12.5% (v/v) glycerol), without added ATP. The reaction was stopped by the addition of 200 μl of proteinase K solution (20 mg/ml stock). After 30 min at 37°C the RNA was phenol extracted, precipitated with ethanol and analysed by electrophoresis on non-denaturing and denaturing gels.

Gel electrophoresis of proteins and RNAs

Phosphorylated proteins were subjected to SDS gel electrophoresis and autoradiography using standard methods. Labelled RNAs were analysed by electrophoresis on either 4% polyacrylamide non-denaturing gels or 5% polyacrylamide/7 M urea

denaturing gels and the dried gels were examined by autoradiography.

Trichloroacetic acid precipitation

To test for possible exonuclease activity of the La antigen aliquots of the radioactive c-myc and reovirus dsRNAs used for the gel analyses described above were precipitated on GF/C glass fibre filters (Whatman) with cold 5% trichloroacetic acid. After three washes with acid and one each with ethanol and acetone the filters were dried and the acid-insoluble radioactivity determined by liquid scintillation counting.

RESULTS

The La antigen increases the dsRNA requirement for activation of PKR

When PKR is incubated with low concentrations of dsRNA in the presence of ATP the protein kinase becomes activated in a step involving autophosphorylation (33, 34). The minimum concentration of the synthetic dsRNA poly(I).poly(C) required for this process is about 10 ng/ml and the optimum concentration lies between 100 and 1000 ng/ml (13, 35). At higher concentrations of dsRNA activation is inhibited, probably because dsRNA-dependent protein dimerization is required for autophosphorylation and this is less likely to occur in the presence of excess dsRNA. Such a dose-response gives rise to a characteristic bell-shaped activation curve (35). When the dsRNA-dependent autophosphorylation of PKR was assayed in the presence and absence of recombinant La antigen we observed a marked inhibition by La (Figure 1). Dose–response curves indicated that inhibition of PKR autophosphorylation was obtained when La was added at 10 μg/ml or more, with a maximal effect at 25 μg/ml (data not shown). The inhibition was associated with a shift in the concentration of poly(I).poly(C) required for the maximal autophosphorylation of PKR from around 500 ng/ml to 5000 ng/ml. Inhibition by La was also observed when the phosphorylation by PKR of its principal natural substrate, the α subunit of initiation factor eIF-2, was assayed (Figure 2). Again there was an increase of at least five-fold in the requirement for dsRNA in the presence of La. Phosphorylation of eIF-2α in the absence of La could be observed at 10 ng/ml of poly(I).poly(C), and the optimum concentration of activator was 500 ng/ml. In contrast, in the presence of La at least 50 ng/ml was needed as the threshold concentration of dsRNA for PKR activity and phosphorylation was still inhibited even when poly(I).poly(C) was

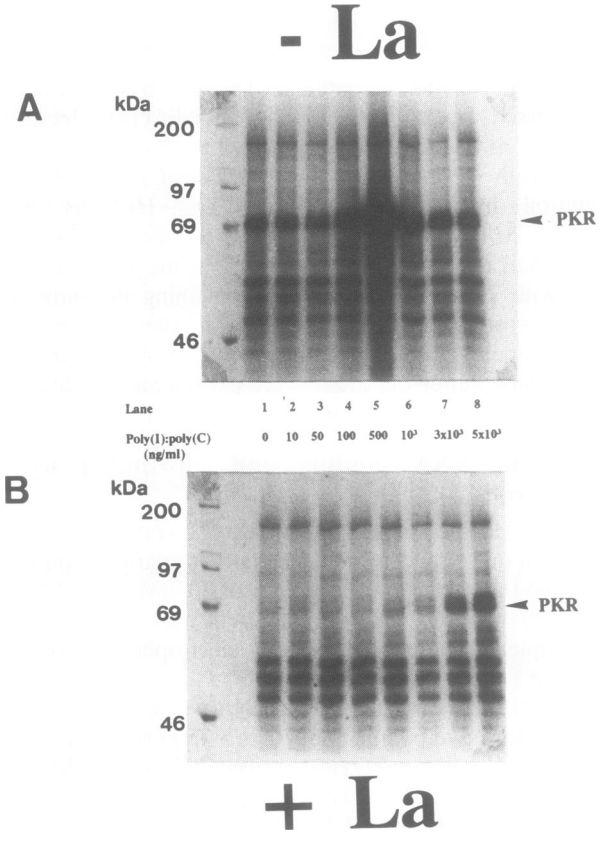

Figure 1. Inhibition of the dsRNA-dependent autophosphorylation of PKR by recombinant La antigen. Partially purified rabbit liver PKR (ca. 4 μg total protein) was incubated with [γ-^{32}P]ATP (170 μCi/ml) and the indicated concentrations of poly(I).poly(C) in the absence (panel **A**) and presence (panel **B**) of recombinant La antigen (25 μg/ml). After 15 min at 30°C the phosphorylated proteins were denatured in SDS sample buffer and subjected to SDS gel electrophoresis and autoradiography. The left-hand track in each panel shows the migration of molecular mass marker proteins (sizes in kDa are indicated). The position of the PKR band which migrates at 68 kDa is shown by the arrow. The bands located at 50–60 kDa are phosphoprotein contaminants in the PKR preparation which provide an indication of the uniformity of loading of the samples.

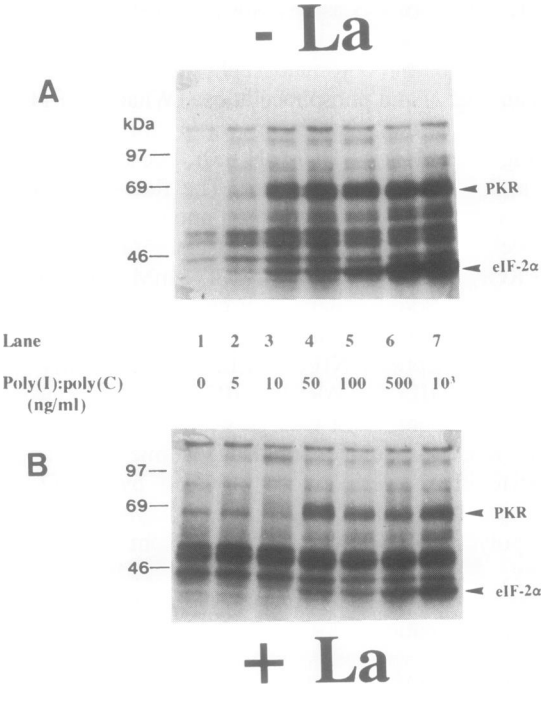

Figure 2. Inhibition of PKR-catalysed phosphorylation of eIF-2α by recombinant La antigen. Partially purified rabbit liver PKR (ca. 4 μg total protein) was incubated with purified initiation factor eIF-2 (50 μg/ml), [γ-^{32}P]ATP (170 μCi/ml) and the indicated concentrations of poly(I).poly(C) in the absence (panel **A**) and presence (panel **B**) of recombinant La antigen (25 μg/ml). After 15 min at 30°C the samples were denatured in SDS sample buffer and subjected to SDS gel electrophoresis and autoradiography. The positions of marker proteins (sizes in kDa) are indicated. The PKR band at 68 kDa and the eIF-2α band at 38 kDa are shown by arrows.

present at 1000 ng/ml (Figure 2). The La antigen has been reported to possess ATPase activity (ref. 5 and M.Bachmann, personal communication). However this is unlikely to account for the observed inhibition of phosphorylation of PKR or eIF-2α since the phosphorylation of other proteins on the gels shown in Figures 1 and 2 was not inhibited by La.

The La antigen inhibits activation but not the activity of PKR

The shift to the right in the dsRNA concentration curve for autophosphorylation of PKR in the presence of the La antigen suggested that the latter was interfering with the dsRNA-dependent activation of the protein kinase. Such an effect could be due to competition for dsRNA binding between the two proteins, or La could be directly inhibiting the protein kinase activity of PKR for both autophosphorylation and the phosphorylation of other substrates. It is possible to distinguish between these alternatives by taking advantage of the fact that PKR only requires dsRNA for its activation whereas the subsequent ability of the protein kinase to phosphorylate exogenous substrates is independent of dsRNA (33). We therefore examined the ability of La to inhibit PKR kinase activity when the La antigen was added either during the dsRNA-dependent activation step or after activation had taken place in a pre-incubation with dsRNA. In this experiment the protein kinase activity of PKR was assayed by the phosphorylation of a peptide corresponding to amino acids 40–59 of eIF-2α. This peptide contains a serine residue at position 51, which is the site of phosphorylation by PKR in eIF-2α itself (36). Table 1 shows that the peptide is a good substrate for phosphorylation by PKR.

Table 1. Phosphorylation of Ser^{51} in a synthetic peptide containing amino acids 40–59 of eIF-2α; evidence that the activation rather than the activity of PKR is inhibited by the La antigen

A.

Time (min)	Radioactivity incorporated: Peptide S51 counts per min (× 10^{-4})	Peptide A51
2	4.68	1.32
10	6.85	1.57
20	10.66	0.31
30	16.69	0.15

B. poly(I).poly(C)

(ng/ml)	Radioactivity incorporated into peptide S51: −La counts per min (× 10^{-3})	+La (during preincubation) (% inhibition by La)	+La (after preincubation)
1	0.46	0.42	0.37
10	3.50	1.69 (52)	2.95 (16)
100	9.99	2.36 (76)	8.26 (17)
1000	9.78	5.46 (44)	7.90 (19)

A: Partially purified rabbit liver PKR was incubated with the synthetic peptides NIEGMILLSELSRRRIRSIN (peptide S51) or NIEGMILLSELARRRIRSIN (peptide A51) (each 1 mM) in the presence of [γ-^{32}P]ATP (150 μCi/ml) and poly(I).poly(C) (500 ng/ml). After the indicated times incorporation of radioactivity into the peptides was assayed as described in Materials and Methods. Zero time counts have been subtracted.

B: In a separate experiment PKR was pre-incubated with the indicated concentrations of poly(I).poly(C) in the presence or absence of recombinant La antigen (25 μg/ml). After 15 min [γ-^{32}P]ATP (150 μCi/ml) and peptide S51 (1 mM) were added. The La antigen (25 μg/ml) was also added to another sample after the pre-incubation step (i.e. at the same time as the ATP and the peptide). Phosphorylation of the peptide was determined after a further 15 min incubation as in panel A. Radioactivity incorporated in the absence of poly(I).poly(C) has been subtracted. The % inhibition by La is shown in parentheses.

The data suggest (but do not rigorously prove) that Ser^{51} is the specific site for this modification, since replacement of this residue with an alanine abolished phosphorylation even though other serines were still present at positions 48 and 57. When PKR was incubated with dsRNA at the optimal concentration (100 ng/ml) in the presence of the La antigen its subsequent ability to phosphorylate the peptide was inhibited by 76%. In contrast, if the PKR was first pre-incubated with this concentration of poly(I).poly(C) the later addition of La had relatively little effect on the activity of the enzyme (17% inhibition). This result suggests that it is the activation rather than the activity of PKR that is sensitive to the La antigen, a conclusion which is consistent with the increased requirement for dsRNA when La is present. The data also indicate that the recombinant La antigen preparation has no significant toxic effect on PKR activity and further rule out the ATPase activity of La as a basis for the inhibition of phosphorylation of the peptide by PKR.

La binds dsRNA and converts it to single-stranded RNA

Since the La antigen appeared to act specifically on the dsRNA-mediated activation of PKR we investigated whether it can bind dsRNA and thus act as a competitor for this ligand. Figure 3 shows that recombinant La binds the synthetic dsRNA, poly(I).poly(C), as assessed by retention of the RNA on a cellulose nitrate filter. The formation of La–dsRNA complexes was also examined by gel shift assays under non-denaturing conditions, using a 940 bp dsRNA prepared by symmetrical *in vitro* transcription of a plasmid containing a portion of the mouse c-*myc* gene (12). One strand of this dsRNA substrate was labelled with ^{32}P during the synthesis of the transcript. Incubation with increasing concentrations of La antigen had two effects on the resulting gel pattern of the radioactive RNA. Firstly, there was a retardation of the dsRNA band in the presence of the protein; the extent of this shift in mobility was dependent on the concentration of La antigen present (Figure 4). More dramatically, there was a substantial conversion of the radioactive substrate into a form which co-migrated with the single-stranded ^{32}P-labelled myc transcript (Figure 4).

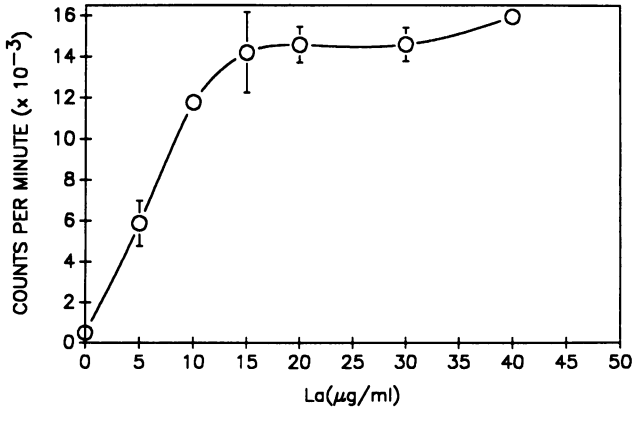

Figure 3. Binding of dsRNA to recombinant La antigen. The indicated amounts of recombinant La were incubated with ^{32}P-end labelled poly(I).poly(C) (2×10^6 counts per min/ml) for 15 min at 30°C. Binding of the radioactive dsRNA to the protein was measured by a cellulose nitrate filter binding assay as described in Materials and Methods. Means ± standard errors of triplicate determinations are shown.

In order to analyse the consequences of La–dsRNA interaction in more detail we also performed experiments in which RNA was deproteinized before subjecting it to gel electrophoresis under both denaturing and non-denaturing conditions. Using naturally double-stranded reovirus genomic RNA which had been end-labelled with [^{32}P]pCp, these assays showed that the La antigen converted essentially all of the RNA to a single-stranded form within 15 min at 30°C (Figure 5, upper panel). Bachmann's laboratory has observed an RNA cleavage activity of La which is directed towards the 3′ single-stranded overhangs of partially double-stranded molecules (M.Bachmann, personal communication). We therefore examined whether any cleavage of the dsRNA substrates occurred in our experiments. The possibility of exonucleolytic activity at the 3′ end was investigated by subjecting an aliquot of the end-labelled reovirus RNA to trichloroacetic acid precipitation but no loss of radioactivity into acid-soluble products was observed over a 60 min incubation period with La (data not shown). Endonuclease activity against the reovirus dsRNA was assayed by subjecting the RNA to electrophoresis under denaturing conditions. This separates several of the viral RNA species and four discrete bands become visible on the gel (Figure 5, lower panel). No changes in size or amounts of these bands were apparent over the 60 min incubation period with La. These results suggest that the La antigen does not modify this dsRNA substrate or cleave it at the 3′ end. Indeed after 60 min we observed some renaturation of the reovirus dsRNA, presumably because the unwinding activity of La begins to decline after this long incubation time. This suggests that RNA denaturation by La is probably reversible.

DISCUSSION

In this paper we have presented evidence that the La antigen has the potential to act as an inhibitor of the dsRNA-activated protein kinase PKR and that this effect is probably due to the ability of La reversibly to unwind dsRNA. Since single-stranded RNA does not bind to PKR, and cannot substitute for dsRNA as an activator of this protein kinase (37), the La antigen most likely acts by reducing the effective concentration of dsRNA available to the enzyme. In accord with this interpretation, increasing the concentration of dsRNA at least partially overcomes the effect of the La antigen on PKR activation. We have also shown that, once activated, the ability of PKR to phosphorylate an exogenous substrate is relatively insensitive to inhibition by La. This provides further support for the view that it is specifically the dsRNA-dependent step in PKR activation that is affected by the La antigen.

Implications of La–dsRNA interactions for the control of protein synthesis

Bachmann *et al.* (5) have previously described a DNA–RNA unwinding activity associated with La antigen preparations from both mouse 3T3 cells and calf thymus, and the same group has recently extended these observations to the identification of an RNA–RNA unwinding activity (M.Bachmann, personal communication). Our results using both an *in vitro*-transcribed 940 bp dsRNA substrate and naturally occurring reovirus dsRNA are in agreement with the conclusion that La shows dsRNA unwinding activity. It is not yet clear what functional relationship such an activity may bear to the effects of other helicases and dsRNA unwinding proteins such as initiation factors eIF-4A and -4F (38–42) or the 'unwinding-modifying' enzyme described in a number of eukaryotic cell types (32, 43–45). We have no evidence that La either cleaves or otherwise modifies the limited range of dsRNA substrates that we have examined.

Meerovitch *et al.* (10) have shown that the La antigen binds to a specific part of the long 5′ untranslated region of poliovirus RNA and may increase the fidelity of initiation of translation on this viral RNA. Such an effect might be due to the ability of the protein to unwind regions of secondary structure locally in the poliovirus 5′ UTR, perhaps in a manner similar to that of eIF-4A during initiation on cellular mRNAs (38–42). La has also been

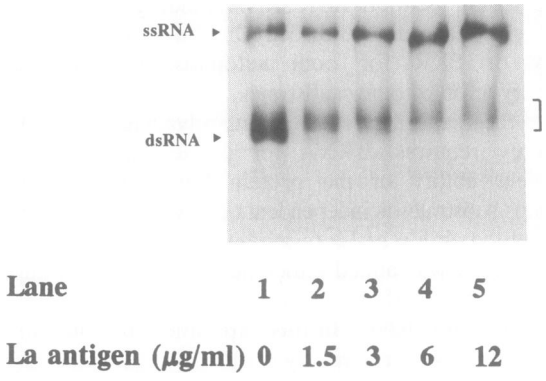

Figure 4. Formation of RNA–protein complexes and conversion of dsRNA into single-stranded RNA in the presence of recombinant La antigen. A 940 bp double-stranded RNA synthesized by transcription from both strands of a fragment of the mouse c-*myc* gene was prepared as described in Materials and Methods. One strand was made radioactive by including [α-^{32}P]UTP in the transcription mix. The dsRNA (2×10^5 counts per min) was incubated with the indicated concentrations of recombinant La antigen in a 25 μl reaction volume for 15 min at 30°C under the conditions described in Materials and Methods. The samples were then analysed by gel electrophoresis under non-denaturing conditions, followed by autoradiography. The single-stranded and double-stranded forms of the RNA were identified from the respective positions of migration on the gel of the radioactive RNA strand alone or after its hybridization to the complementary unlabelled strand. The bracket on the right indicates retarded dsRNA complexes formed in the presence of increasing concentrations of the La antigen.

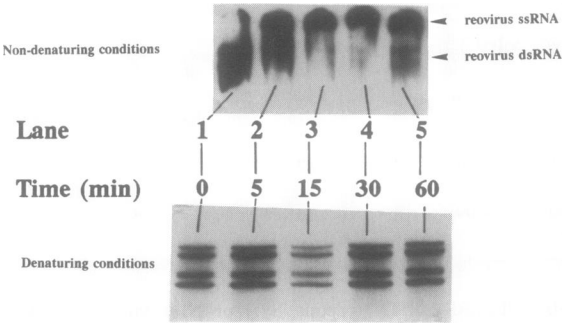

Figure 5. La antigen unwinds but does not cleave reovirus dsRNA. End-labelled reovirus genomic dsRNA (ca. 2×10^5 counts per min per reaction) was incubated with recombinant La antigen (25 μg/ml) at 30°C for the times indicated as described in Materials and Methods. After digestion with proteinase K the samples were phenol extracted and the RNA subjected to gel electrophoresis under both non-denaturing conditions (upper panel) and denaturing conditions (lower panel), followed by autoradiography. The positions of migration on the non-denaturing gel of the double-stranded and single-stranded forms of the reovirus RNAs are indicated.

shown to bind to a number of other viral RNA species (46–50), although no other effects on translation have so far been reported. Our data suggest that, in addition to its possible effects on polypeptide chain initiation on specific mRNAs involving *cis*-acting regions of secondary structure, the La antigen may regulate overall rates of protein synthesis under circumstances where PKR is activated in *trans* by viral (or cellular) dsRNA molecules.

RNA-binding characteristics of La and PKR

Since the La antigen has the potential to regulate PKR by the mechanisms indicated above, it is intriguing that these two proteins not only both bind dsRNA but also have the ability to interact with several other small RNA species in common. A number of RNA polymerase III transcripts that bind to La, including adenovirus VA_I RNA and Epstein–Barr virus RNAs EBER-1 and EBER-2, have also been shown to bind to PKR and to block its activation by dsRNA (reviewed in references 4 and 11). In addition, the small cellular Y RNAs that are components of the cytoplasmic Ro RNP particles (2) also interact with PKR in an apparently similar manner (G.Pruijn and T.Sharp, unpublished observations). In the case of PKR the small RNAs bind to the same site as dsRNA [probably involving amino acids 11–77 and 101–167 (51)] and compete with dsRNA when present at high concentrations *in vitro* (14).

The region of the La antigen that binds single-stranded RNAs (including the small viral RNAs) involves the RNP-80 motif in the N-terminal half of the molecule (residues 112–187) (2, 52–55). This sequence is necessary (although it may not be sufficient) for RNA binding. The nature of the binding site for dsRNA on La has not yet been determined, but amino acid sequence comparisons with PKR have indicated two regions of limited similarity, comprising amino acids 249–271 and 338–358 of La (4). The latter region (GKGKGNKAAQPGSG-KGKVQFQ in human La and GKGKGNKAAQAGSAKGKV-QFQ in bovine La) has features in common with a consensus sequence (GxGxSKKxAKxxAAKxALxxL) found in a number of dsRNA-binding proteins (corresponding to residues 55–75 and 145–161 in human PKR) (51, 56). It also overlaps with a motif (GXXXXGK) at positions 333–339 that is similar to an ATP- or GTP-binding motif (A/GXXXXGKT) found in several DNA and RNA helicases (38,57). Bachmann *et al.* (5) have reported that La possesses DNA–RNA-dependent or dsRNA-dependent ATPase activity and it is possible that the GXXXXGK motif may be required for this activity. However the region with similarity to the dsRNA-binding consensus sequence lies in the C-terminal half of the protein and is quite distinct from the RNP-80 motif. Mutational studies will be necessary in order to establish the relative importance of these two regions for the dsRNA binding and unwinding activity of the La antigen. Further studies are also required to establish the ATP-dependence of the dsRNA unwinding activity of La, since we observed this activity in the absence of added nucleotide. However we cannot exclude the possibility that some ATP remained associated with the La preparation or with the RNA substrates used in the assays.

The precise structural features required in different RNA molecules that permit binding to the La antigen remain to be defined. In the case of the small RNAs an oligo(U) stretch at the 3′ terminus is important (58–62), and binding to such a region may be involved in La-mediated termination of RNA polymerase III transcription (6,7). In molecules such as VA_I RNA and the EBERs the 3′oligo (U) is not involved in base-pairing interactions with other parts of the RNA and has nothing obviously in common with the dsRNA substrates with which La also interacts. It therefore seems probable that quite distinct RNA structural features are required for the dsRNA-binding function of La. Whether these features are also present in the small RNAs remains to be determined; however these molecules all contain large amounts of secondary structure (4, 11) and this may facilitate their interaction with the La antigen.

Does the La antigen regulate PKR *in vivo*?

The most important question raised by the findings in this paper is whether the La antigen has a role to play in the regulation of the protein kinase activity of PKR in either uninfected or virus-infected cells. It is highly likely that PKR is involved in mediating both antiviral and antiproliferative effects of the IFNs and that it acts principally by phosphorylating the α subunit of the eukaryotic protein synthesis initiation factor eIF-2 (17). The importance of PKR in the antiviral effects of the IFNs is indicated by the fact that a number of viruses have evolved mechanisms for blocking the activation of this protein kinase (11, 26). It may be important for uninfected cells also to regulate PKR under various conditions and several cellular inhibitors of PKR have been described (26, 63–66). However it is not known if these are functional in uninfected cells and the modes of action of these molecules have not been established. The ability of the La antigen to block PKR activation by unwinding dsRNA activators may constitute a novel mechanism for regulation of the protein kinase that is distinct from the other cellular or virally mediated pathways so far identified.

If the La antigen has a physiological role in PKR regulation the relative subcellular locations of the two proteins are clearly relevant. PKR has been shown to be largely a cytoplasmic, ribosome-associated protein in IFN-treated cells (67, 68). However, recent evidence indicates that it can also be found in the cell nucleus and nucleolus (15), the ratio of ribosomal PKR to nuclear PKR being about 22:1 in control Daudi cells (16). Moreover, the La antigen, although predominantly a nuclear protein, has also been found in the cytoplasm where it occurs in the Ro RNP particles (2, 55, 69). Recent studies using enucleated cells (70) have confirmed the cytoplasmic presence of La. It is quite possible therefore that PKR and La co-exist in the same locations in the cell, where they may compete for regulatory dsRNA and small RNA ligands. It is of interest that the levels of La antigen, as well as the proportion found in the cytoplasm, have been reported to increase in both virus-infected and transformed cells (8, 71–73). These are physiological situations where the anti-viral and tumour suppressor functions of PKR respectively might be expected to be decreased and it is tempting to speculate that the La antigen could play a role in such regulation. Further studies are now needed to determine how the La and PKR systems interact *in vivo*, and to examine whether the various small RNA species that can bind to both proteins have a part to play in a complex network of control that influences the rate of protein synthesis and/or cell growth.

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