Biochemical analysis and scanning force microscopy reveal productive and nonproductive ADAR2 binding to RNA substrates

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ABSTRACT

Scanning force microscopy (SFM) can be used to image biomolecules at high resolution. Here we demonstrate that single-molecule analysis by SFM complements biochemical data on RNA protein binding and can provide information that cannot be obtained by the usual biochemical methods. We have used this method to study the interaction between the RNA editing enzyme ADAR2 and RNA transcripts containing selective and nonselective editing sites. The natural selectively edited R/G site from glutamate receptor subunit B (GluR-B) was inserted into an RNA backbone molecule consisting of a completely double-stranded (ds) central part and incompletely paired ends derived from potato spindle tuber viroid (PSTVd). This molecule was efficiently edited at the R/G site, but promiscuous editing occurred at nonselective sites in the completely double-stranded region. The construct was also used to analyze binding of ADAR2 to wild-type and modified R/G editing sites in relation to binding at other nonselectively edited sites. Editing analysis together with SFM allow us to differentiate between binding and enzymatic activity. ADAR2 has been reported to have a general affinity to dsRNA. However, we show that there is a prominent bias for stable binding at sites selectively edited over other edited sites. On the other hand, promiscuous editing at nonselective sites apparently results from transient binding of the enzyme to the substrate. Furthermore, we find distinct sites with nonproductive binding of the enzyme.

Keywords: SFM; adenosine deamination; RNA-binding protein; double-stranded RNA; RNA editing; ADAR

INTRODUCTION

Adenosine deaminase that acts on RNA (ADAR) is a double-stranded RNA (dsRNA) binding enzyme that converts adenosine (A) to inosine (I) within structured RNA (for reviews, see Gerber and Keller 2001; Keegan et al. 2001). Two functional ADAR enzymes that share a common modular domain organization, ADAR1 and ADAR2, have been characterized in mammals. These enzymes recognize the double-stranded structure formed by the editing site and a complementary sequence, rather than a consensus sequence. What appears to be promiscuous deamination, in which as much as 30% of the adenosines can be edited, has been seen in the context of long stretches of completely base-paired RNA molecules of noncoding RNA (Morse and Bass 1999; Morse et al. 2002). Selective editing of specific

sites has been found in substrates where base-paired regions are interrupted by mismatches or loops. The editing activity has been detected in many metazoans, but a relatively small number of substrates that possess site-selective editing has been found. In most of these cases, editing of a single adenosine changes the coding capacity of a pre-mRNA and results in an altered protein with properties different from the one encoded by the nonedited RNA. The editing event causes a functional A-to-G replacement because inosine is translated as guanosine. Site-selective A-to-I editing has mainly been found in pre-mRNAs encoding neurotransmitter receptors in mammals and *Drosophila melanogaster* (Sommer et al. 1991; Lomeli et al. 1994; Burns et al. 1997; Palladino et al. 2000). The best-understood example of RNA editing by adenosine deamination occurs in the pre-mRNA of mammalian ionotropic AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) glutamate receptor subunits (GluRs) (for review, see Seeburg et al. 1998). The pre-mRNA of the GluR-B subunit is edited at two sites, Q/R and R/G. The R/G site, located in an arginine codon, is converted to encode for glycine after editing. Editing at the R/G site is developmentally regulated, changing

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the gating kinetics of the receptor (Seeburg et al. 1998). ADAR2 efficiently deaminates the R/G stem-loop of the GluR-B pre-mRNA in vitro (Melcher et al. 1996; O'Connell et al. 1997; Öhman et al. 2000). The enzyme has two double-stranded RNA binding motifs (dsRBMs) and a C-terminal catalytic deamination domain. The dsRBMs have been found in a variety of other functionally diverse proteins such as helicases, kinases, and endoribonucleases (for review, see Fierro-Monti and Mathews 2000). Common to all these proteins are that they bind highly structured and completely double-stranded RNA (dsRNA) with little sequence specificity. Recent studies indicate that the edited base is flipped out of the duplex during the modification process (Stephens et al. 2000). Furthermore, it has been suggested that the RNA binding domain assists the base-flipping by increasing the conformational flexibility of the nucleotides opposite to the edited site (Yi-Brunozzi et al. 2001).

To better understand what determines selective A-to-I editing, we have examined ADAR2 binding to single molecules of highly structured RNA using scanning force microscopy (SFM). We and others have previously used SFM to analyze protein–nucleic acid interactions; for example, Hansma et al. (1999) performed a functional analysis of polymerases on their respective templates by SFM; Herbert et al. (1998) showed that the α -Z domain of ADAR1 binds to Z-DNA; Kasas et al. (1997) were able to monitor the dynamics of *Escherichia coli* RNA polymerase in the SFM; and we (Bonin et al. 2000) have determined preferential binding sites of a protein on dsRNA.

The RNA molecule used in this study contains both a region subjected to site-selective editing and sections that allow promiscuous, nonselective editing. Using this target molecule, it was possible to determine and quantify, editing at the selective R/G editing site in relation to nonselective editing within the same molecule. In addition, we have analyzed ADAR2 binding independent of editing by scanning force microscopy (SFM). By using SFM we demonstrate, in a unique way, that there is a difference in binding for a selectively edited site and a nonselective editing sites. Furthermore, a correlation between efficiency of editing and selective binding could be seen.

RESULTS

The RNA molecule used in the experiments described here can be divided into three parts; one part containing the specific R/G editing site derived from the glutamate receptor pre-mRNA; sec-

ondly, a long region of completely base-paired sequence that is a target for promiscuous editing; and a third part of the RNA that is double-stranded with interruptions of bulges and loops but does not contain a specific editing site (Fig. 1). Apart from the wild-type R/G stem-loop, two mutants were used in this analysis. In the C42U46 mutant sequence, two point mutations changed an unpaired G–G and A–C to a G–C and A–U Watson-Crick base pair, closing two loops in the stem. The other mutant (G56) has the unpaired C exchanged for a G residue opposite the editing site. Short RNAs containing the wild-type and C42U46 sequences have previously been investigated by editing assays, gel mobility shift, and footprinting assays (Öhman et al. 2000). In this investigation, ADAR2 displayed no apparent difference in binding between wild-type and mutant RNA. However, the editing efficiency of the RNA decrease dramatically in the order wild type > C42U46 > G56 (see also Fig. 5 below and Table 1). According to the footprinting data, the association of the protein to the RNA was less pronounced in the C42U46 mutant than in the wild type (Öhman et al. 2000). The R/G stem-loops, wild type and mutants, were inserted into the gus-PSTVd backbone (Fig. 1; Bonin et al. 2001). This backbone RNA was specifically designed for RNA binding proteins, allowing a comparison between selective and promiscuous binding. The transcripts of the backbone RNA and the gus-PSTVd-R/G 75 were used in gel mobility shift assays (Fig. 2A,B). No significant difference in protein binding was detected by this method: the empty backbone bound ADAR2 with approximately equal

FIGURE 1. Structure of the gus-PSTVd RNA molecule with the inserted R/G stem-loop. The enlargement shows the bifurcation with the R/G stem-loop on top. Mutations in the G56 and C42U46 variants are indicated by arrows. The gus and PSTVd parts are indicated. The backbone alone displays a straight rod in m-fold (data not shown; see Bonin et al. 2001). With the insertion of the GluR-B R/G sequence, 9 nt of the backbone 5' and 15 nt 3' of the insert are included in the stem-loop structure. The size of the chimeric RNA is 1007 nt.

TABLE 1. ADAR2 binding and editing of gus-PSTVd RNA constructs

	Binding to R/G stem-loop	Binding to segment 1 ^a	Binding to backbone (not segment 1)	Editing specifically at R/G site	Number of nonselective editing sites ^b
R/G wt	94%	4%	2%	86%	15
C42U46	83%	12%	5%	ND	ND
G56	67%	22%	11%	35%	17

^aBinding to segment 1 is listed separately because this may be enhanced by the presence of the 5' and 3' ends of the molecule in this region.

^bFor determination of nonselective editing sites, 170 A residues in the backbone molecule were analyzed. (ND) Not determined.

efficiency as the RNA with the inserted R/G stem-loop. To ensure that the shift was not caused by conformational changes due to editing (a less double-stranded character of the substrate), the gus-PSTVd RNA was treated with phenol to remove the ADAR2 protein after the binding. This RNA (post-phenol) migrated like the free RNA after phenol treat-

ment, indicating that the shift was caused by protein binding. A slight shift observed at 1.7 nM ADAR2 post-phenol treatment probably indicates slower migration because of editing. As previously seen, the short substrate containing only the R/G stem-loop exhibited two defined complex bands (Fig. 2C), which have been suggested to represent binding of protein monomers and dimers (Jaikaran et al. 2002).

In scanning force microscopy, the gus-PSTVd substrates displayed a structure resembling that predicted by m-fold (Zuker 2000). The backbone alone appeared like a rigid rod (Bonin et al. 2001), whereas insertion of the editing sites resulted in the expected asymmetric branching that allow an orientation of the molecule (see Fig. 1). The three different target molecules were used to determine relative protein binding to the wild type and mutated R/G binding sites in respect to the backbone. As described previously (Bonin et al. 2001), the 130-nm-long RNA molecule was subdivided into 10 sections of 13 nm each. Segments 1 and 2 as well as 8 to 10 contain the partially double-stranded PSTVd RNA, whereas segments 3 to 7 contained the completely paired gus RNA. The R/G stem-loop structure was located in segment 8. ADAR2 showed an average diameter of 13 nm and an average height of 1 nm (but see below). Bound molecules could thus be assigned to a specific section. As discussed below, it was not possible to determine absolute binding (i.e., the ratio of free protein to RNA-associated protein) in a meaningful way because it appeared that free protein was preferentially lost in the washing steps. Figure 3 shows representative SFM images of the backbone molecule and of gus-PSTVd-R/G75 incubated with ADAR2. The rod-shaped backbone molecule (Fig. 3A) is symmetrical, and an orientation cannot be determined. With gus-PSTVd-R/G75, the bifurcation of the RNA is readily detectable (Fig. 3B), especially in free RNA molecules. In many cases it seems that the stem-loop structure is covered or condensed by ADAR2 binding.

In Figure 4, the relative binding frequencies of ADAR2 are shown for the backbone gus-PSTVd and the three molecules containing the R/G stem-loop variants. The backbone alone was not oriented; the bottom-left end of the RNA in the SFM image was arbitrarily assigned to segment 1. This resulted in apparently symmetric binding frequen-

FIGURE 2. Gel mobility shift (GMS) assay with gus-PSTVd-substrates and with the 75-nt R/G site stem-loop structure. (A) GMS assay with gus-PSTVd-R/G75. Increasing amounts of recombinant ADAR2 were added as indicated. A shift to a higher-molecular-weight band was found at 0.17 nM protein. (B) GMS assay with gus-PSTVd; the amount of ADAR2 added is indicated. In lanes marked as “post phenol,” the samples were treated with phenol after protein binding (see Materials and Methods). Lanes marked as “post editing” indicate an editing assay prior to the binding assay. (Lane C) A control with nonedited free RNA. (C) GMS assay with a 75-nt-long R/G site stem-loop structure. Increasing amounts of recombinant ADAR2 were added as indicated.

FIGURE 3. SFM images of ADAR2 binding to different substrates. (A) SFM image of gus-PSTVd backbone molecules with bound ADAR2 proteins. (Right panel) A single molecule is enlarged. Protein binding can be readily seen on the right end of the RNA. (B) SFM image of gus-PSTVd-R/G75 molecules with bound ADAR2 proteins. Note the bifurcation of the RNA, which was used to determine the orientation of the molecule. Yellow arrows indicate RNAs with ADAR2 bound to the editing stem-loop; blue arrows indicate RNAs without bound ADAR2. Note that in all images the background of unbound protein is very low. Images show selected frames.

cies along the molecule (Fig. 4A). From this graph we could not conclude if one or both ends of the PSTVd part had a binding preference for ADAR (but see below). However, the double-stranded gus RNA displayed very limited binding of the protein: only a few molecules were found on segments 3–7. A gel mobility shift assay was done to ensure that the limited binding shown by SFM was not due to the fact that editing in the double-stranded region of the backbone RNA had made the substrate too single stranded by I:U mismatches to be bound by ADAR2. Pre-edited backbone gus-PSTVd RNA was shifted by ADAR2 approximately as well as nonedited RNA, indicating that the partially modified substrate could still bind the protein efficiently (Fig. 2B, right panel).

When the R/G editing substrate was introduced into segment 8, this was in all cases the preferential binding site for the enzyme. Binding to other parts of the backbone was marginal and mostly restricted to the distal end (segment

1). We therefore concluded that most of the end binding to the backbone molecule without a selectively edited site also occurred in segment 1. In substrates containing the wild-type R/G stem-loop, 94% of the protein molecules were found at the selective R/G editing site; with the C42U46 mutations this was reduced to 83%, whereas the G56 mutant could only recruit 67% of ADAR2. Consequently, binding to the backbone increased. This was not only because of the known preferential binding to the 5' and 3' ends located in segment 1 (see also Discussion), but also because of an overall increased binding: the percentage of enzyme bound to internal segments (excluding segment 1) increased from 2% (R/G wild type) to 5% (C42U46) to 11% (G56; see also Table 1). Binding to the backbone was low and therefore statistically difficult to evaluate, but there was no obvious preference for either dsRNA in the gus section or partially unpaired RNA in the PSTVd section (except for segment 1).

An analysis of the *in vitro* editing efficiency at the R/G site in gus-PSTVd constructs was made using limited primer extension. The molecule with the wild-type R/G sequence showed an efficient R/G editing of 86%, and introduction of the G56 mutation decreased editing to 35% (Fig. 5). Editing of the wild-type R/G site agreed with the binding at segment 8, including the R/G stem-loop. However, the editing efficiency of the mutated R/G sites appeared quantitatively lower than the observed ADAR2 binding at the respective sites.

To detect nonselective editing by ADAR2 and to be able to compare binding with editing in the same molecules, the substrates used in the SFM analysis were incubated with ADAR2, amplified by reverse transcriptase, followed by a polymerase chain reaction (RT-PCR), and then sequenced. Adenosine-to-inosine modifications were determined by either heterogeneous A and G peaks or single G peaks on the electropherogram after sequencing. Nonselective editing was detected in segments 3–7 (Table 2). In this area the gus RNA is completely double-stranded, and promiscuous A-to-I editing would be expected. However, binding to these segments was infrequent. In contrast, no editing was found in the only partially double-stranded PSTVd sections of the backbone. Segment 1 showed the highest nonselective binding preference in the SFM images; however, no editing at all was detected in this region. Sites of nonselective editing were compared between the wild-type R/G gus-PSTVd transcript, the G56 mutant, and the backbone gus-PSTVd sequence alone. The editing pattern was very similar in the three transcripts, but by using this method the efficiency of editing at individual sites is difficult to determine accurately.

DISCUSSION

To investigate the structural requirements for ADAR editing, most studies have used glutamate receptor pre-mRNA

FIGURE 4. Distribution of ADAR2 binding on the different target molecules. (A) Binding to the gus-PSTVd backbone RNA. The orientation of the molecule was arbitrarily determined (see Materials and Methods), therefore the distribution of binding appears symmetrical. ADAR2 binding to the backbone (B) with the R/G 75 (wild-type) stem-loop inserted, (C) with the C42U46 stem-loop inserted, and (D) with the G56 point mutation stem-loop inserted. (B–D) The orientation was unambiguously determined by the bifurcation on the *right* end of the molecule (Fig. 1). Bound protein was assigned to one of 10 segments (numbered from *left* to *right*) of the RNA molecule. Except for two cases, only one protein was bound to the RNA. (*n*) Number of RNA molecules examined.

with mutated and wild-type sequence to compare binding and editing efficiency. However, as exemplified in this paper, this neglects the following points: First, editing and binding are separate events; a site may very well be recognized for binding but still be edited with no or very low efficiency. Second, the enzyme is forced to a single substrate, and there is no competition with potential binding sites on other substrates. This is particularly important because ADAR2 apparently can bind and edit any completely double-stranded RNA.

To determine binding and editing requirements in a large molecule containing both a genuine editing site and sequence with no known editing sites, we have used the backbone gus-PSTVd RNA sequence fused to the sequence of the selectively edited R/G site of GluR-B pre-mRNA. In a unique way, the competition between ADAR2 binding to selectively and promiscuously edited sites can be analyzed by SFM. We show that >90% of the molecules preferentially bind to segment 8, where the wild-type R/G site is situated (Fig. 4B). This implies that although ADAR2 binds and

edits any double-stranded RNA, the binding to selectively edited sites is more frequent. It cannot, however, be determined if the observed binding occurs on the substrate (nonedited site) or the product (edited site) because the binding affinities of these molecules are very similar (Fig. 2B; Öhman et al. 2000). Binding of the R/G stem-loop determined by SFM correlated well with selective R/G editing. When the efficiency of selective editing was decreased by mutations in the vicinity of the R/G site, binding was less prominent. With the mutations, an increased binding to the first segment of all RNA molecules was observed. We have previously shown that the ends of dsRNA are preferential binding sites for different proteins with affinity to RNA (Bonin et al. 2000, 2001). Increased binding to segment 1 (shown in Fig. 4B–D) might therefore be explained by the location of the 5' and 3' ends of the RNA in this region. No A-to-I editing at all was observed in this area, indicating that the increased binding observed in this region is non-productive. The experiments shown in Figure 4 neglected the absolute affinity of the protein for the RNA. One could

TABLE 2. Nonselective editing of backbone gus-PSTVd RNA constructs

	Segment number									
	1	2	3	4	5	6	7	8L	9	10
gus-PSTV backbone substrate										
No. edited sites	0	0	2	1	2	5	6	0	0	0
Total no. of As analyzed	12	25	21	15	21	20	21	14	18	3
Percent edited As	—	—	10	7	10	25	29	—	—	—
Wild-type R/G substrate										
No. edited sites	0	0	2	1	2	5	5	0	0	0
Total no. of As analyzed	12	25	21	15	21	20	21	14	18	3
Percent edited As	—	—	10	7	10	25	24	—	—	—
G56 mutant substrate										
No. edited sites	0	0	3	1	2	5	6	0	0	0
Total no. of As analyzed	12	25	21	15	21	20	21	14	18	3
Percent edited As	—	—	14	7	10	25	29	—	—	—

The numbers of editing sites in both *upper* and *lower* strands were combined unless indicated (L = *lower* strand only). Editing was defined as an unambiguous heterogenic A and G peak in the sequencing electropherogram.

assume that with molecules containing low-affinity binding sites, the amount of free enzyme would be larger. However, it turned out to be difficult to test this experimentally because we have no means to determine if the RNA, the free protein, or the complex has a higher affinity to the mica surface and preferentially remains attached after the washing step in the preparation of the samples. Experiments using different ADAR2 concentrations (data not shown) indicate that unbound protein is more easily washed off. Alternatively, ADAR2 may form dimers at higher concentrations, which cannot be unambiguously distinguished from monomers. We observed a distribution of protein height from 0.5 nm to 2.5 nm with a broad maximum from 0.6 nm to 1.4 nm, but a bimodal distribution, indicative for monomers and dimers, was statistically not significant (data

not shown). It appears likely that ADAR2 has an asymmetric shape, and different positioning on the surface obscures the detection of distinct dimers. However, the overall increase in the height of the protein particles with increasing protein concentrations indicated dimerization rather than nonspecific aggregation, because we never observed large complexes even at the highest protein concentrations. This is in agreement with the observation in gel mobility assays that are indicative for a stepwise formation of a monomer and then a dimer at an editing site (see Fig. 2C; Jaikaran et al. 2002).

In the experiments presented here, on average 50% of the RNA molecules exhibited binding to the enzyme. Interestingly, even with fourfold higher concentrations of protein, RNA molecules with more than one ADAR molecule (or dimer) attached were rarely found. With a total of almost 1000 RNA–protein complexes evaluated in this study, only two RNAs show binding at two sites (data not shown). It therefore seems that binding of one enzyme inhibits the entry of a second one even over a large distance.

Site-selective editing at the R/G site was shown to correlate with the binding observed in the SFM analysis. This indicates that site-selective binding leads to productive editing. However, nonselective editing of the transcripts does not correlate with binding. For example, in the completely double-stranded region of segment 7, a high extent of nonselective editing was detected at several adenosines, but few ADAR molecules were found to bind to this segment. On the contrary, in segment 1, but also in segments 2, 9, and 10, significant binding in the absence of editing was observed. These are surprising examples of nonproductive binding. In summary, we can distinguish three types of binding/editing sites: (1) the cognate editing sites, where binding and editing correlate; (2) promiscuous hit-and-run editing sites, where little binding but significant editing is observed; and

FIGURE 5. Editing at the R/G site in the wild-type and G56 mutant substrates. A limited primer-extension assay was used to monitor the R/G site after incubation without rADAR2 (C) or with rADAR2. The assay was performed on the wild-type (wt R/G) and the G56 mutant (G56) substrates. Primer extension was done in the presence of ddT so that reverse transcriptase terminated at the R/G site for nonedited molecules and continued to the next adenosine if the R/G was edited. Editing efficiency was calculated as described previously in Öhman et al. (2000).

(3) nonproductive binding sites, where no editing is found even though significant binding is detected. Particularly the hit-and-run editing sites would pose a problem in vivo and result in misediting, which may not be tolerated by an organism. ADAR2 readily recognizes specific editing sites, and thus a guidance to such sequences appears not to be necessary. It would rather be required to implement a mechanism to inhibit promiscuous editing at other sites. Given that binding to nonselective sites appears to be transient, one could envision a cofactor that further destabilizes interactions with noncognate sequences and thus avoids misediting. It should be noted that binding observed by SFM does not necessarily reflect the affinity of the protein to a substrate: the off-rate that is apparently different for selective and nonselective sites is not taken into consideration in this analysis. Another explanation for the apparently increased binding to the backbone in the mutated substrates could be that the enzyme actually enters the RNA molecule at a more or less defined site in vitro and then scans for an appropriate editing site. With no such site available (or a weak editing site as in the G56 mutant), the SFM snapshot would show more randomly distributed, scanning proteins in addition to those attached to preferential sites like G56 or the molecule ends in segment 1.

Our analysis demonstrates the power of the SFM–biochemistry combination in understanding molecular interactions: the finding of productive, nonproductive, and hit-and-run ADAR binding sites provides new views on editing by ADAR2 and may open new ways to investigate the question of how misediting is avoided in vivo.

MATERIALS AND METHODS

Construction of vectors for in vitro transcription

Complementary single-stranded oligonucleotides were synthesized by Life Technologies. The following synthetic oligonucleotides with the sequence 5' to 3' were used: R/G75, GGATCCTCATT AAGTGGGTGAATAGTATAACAATGTGCTCAATGTTGTTAT AGTATCCCACCTACCCTGATG; C42U46, GGATCCTCATTAAGTGGGTGGAATGATATAACAATGTGCTCAATGTTGTTATATCTATTCCACCTACCCTGATG; G56, GGATCCTCATTAAGGTGGTGAATAGTATAACAATGTGCTCAATGTTGTTATAGTATCCCACCTACCCTGATG.

The complementary oligonucleotides were annealed and 5'-phosphorylated with T4 polynucleotide kinase, according to standard procedures (Maniatis et al. 1982). The plasmid pT3T7 gus-PSTVd (Bonin et al. 2001) contains a single *Sma*I restriction site. After restriction digestion, the plasmid was dephosphorylated with shrimp alkaline phosphatase, according to standard procedures (Maniatis et al. 1982). The annealed modified oligonucleotides were cloned into this *Sma*I site using T4 DNA ligase and the restriction enzyme-controlled ligation. Recombinant plasmids were transformed into *E. coli* DH5 α cells. Plasmids were selected by restriction digestion and confirmed by sequencing. The resulting constructs were the plasmids pT3T7gus-PSTVd R/G75, pT3T7gus-PSTVd C42U46, and pT3T7gus-PSTVd G56.

The gus-PSTVd R/G75, C42U46, and G56 RNAs were synthesized by in vitro transcription with T7 RNA polymerase from plasmids linearized with *Sal*I. The RNA was purified by phenol/chloroform extraction and ethanol precipitation.

Gel mobility shift assays

Samples for gel-retardation assays using the gus-PSTVd-constructs were prepared by incubating 170 pM radioactively labeled RNA and 30 pM–3.4 nM ADAR2 in 10 mM Tris-HCl (pH 8.0), 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 10% glycerol, 0.1 mg/mL BSA, and 0.2 mM ATP in a total volume of 15 μ L at room temperature for 15 min (Bass et al. 1994). The complexes were analyzed on a nondenaturing 5% PAGE at 4°C in 1 $\times$ TBE for 2 h at 160 V. Samples labeled “post phenol” were prepared as above but extracted with 1 volume of phenol/chloroform after ADAR2 binding. The “post editing” samples, were edited according to the ADAR editing assay (see below) prior to the gel-retardation assay described above. The gel-retardation assay for the R/G 75 stem-loop structure was prepared by incubating 10 pM RNA and 15 pM–28 nM ADAR2 (described previously in Öhman et al. 2000).

Preparation of samples for SFM and imaging parameters

Mica was freshly cleaved and activated for 1 min in an air plasma at 0.2 mbar, 600 V, and 20 kHz. Then 300–330 nM dsRNA was incubated with 10–75 nM ADAR2 in 10 mM Tris-HCl (pH 8.0), 25 mM KCl, and 10 mM NaCl at room temperature for 2 min. Next, 1 μ L of this assay was loaded onto the mica surface together with 9 μ L of 5 mM MgCl₂. After 1 min, the mica was rinsed with 1 mL of water and blown dry with nitrogen. Samples were scanned with Nanoscope III multimode SFM (Digital instruments) operated in the tapping mode using an E scanner with a 10 $\times$ 10 (x, y) $\times$ 2.5 (z) μ m scan range. Microfabricated silicon tips with a force constant of 42 N/m and a resonance frequency of 320 kHz (Pointprobes, Nanosensors GmbH) were used. Images were obtained in the topographic mode. Measurements were done in air (relative humidity 25%–60%) at room temperature (18°C–27°C). Images (512 $\times$ 512 pixels) were taken at 1 Hz scanning frequency. Images were processed with Nanoscope software including operations of plane fitting and flattening. Contour length measurement was done by drawing a traverse line along the skeleton of the molecule on the screen. For quantitative analysis of ADAR2 binding to the RNA molecules, molecules were subdivided into 10 segments. Left and down in the image was arbitrarily defined as segment 1 for gus-PSTVd. RNA molecules containing an editing site could be unambiguously oriented by the bifurcation. Segment 1 was assigned to the distal end with respect to the bifurcation/editing site. The number of ADAR2 molecules found in each segment was counted and divided by the total number of bound proteins (relative binding frequency).

Protein expression

Recombinant rat ADAR2a protein used in the binding and editing assays was expressed in *Pichia pastoris* (previously described in Öhman et al. 2000).

ADAR editing assays

For editing determination by sequencing, 500 fmole of RNA was dissolved in 40 mM Tris-HCl (pH 8), 5% glycerol, 25 mM KCl, 1.1 mM MgCl₂, 1 mM DTT, 5 mM EDTA, 0.2 mM ATP, and 0.16 U/μL RNasin (Promega). Then 200 ng of recombinant rat ADAR2 was added to a total volume of 100 μL and incubated at 30°C for 30 min. Edited RNA was phenol/chloroform-extracted and precipitated. Using Omniscript reverse transcriptase (QIAGEN), the edited RNA was transcribed according to the recommended protocol, except for the following. Because of the high degree of secondary structure, the RNA and primer mix was denatured at 65°C for 5 min. Elongation incubation was prolonged to 2 h, and the temperature was elevated to 44°C. Using *Taq* DNA polymerase (QIAGEN) with the optional Q-solution for difficult templates, the cDNA was amplified in four overlapping sections. The primer sequences are available from the author. The PCR fragments were sequenced using the BigDye sequencing kit (Applied Biosystems) and run on an ABI PRISM 377 DNA Sequencer.

A limited primer extension assay was performed to determine editing at the R/G site (previously described in Öhman et al. 2000).

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