Limited Proteolysis as a Probe of the Conformation and Nucleic Acid Binding Regions of Nucleolin†

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ABSTRACT: Nucleolin, also called protein C23, is a RNA-associated protein implicated in the early stages of ribosome assembly. To study the general conformation and map the nucleic acid binding regions, rat nucleolin was subjected to limited proteolysis using trypsin and chymotrypsin in the presence or absence of poly(G). The cleavage sites were classified according to their locations in the three putative domains: the highly polar amino-terminal domain, the central nucleic acid binding domain, which contains four 90-residue repeats, and the carboxyl-terminal domain, which is rich is glycine, dimethylarginine, and phenylalanine. The most labile sites were found in basic segments of the amino-terminal domain. This region was stabilized by Mg^{2+}. At low enzyme concentrations, cleavage by trypsin or chymotrypsin in the amino-terminal domain was enhanced by poly(G). Trypsin produced a relatively stable 48-kDa fragment containing the central and carboxyl-terminal domains. The enhanced cleavage suggests that binding of nucleic acid by the central domain alters the conformation of the amino-terminal domain, exposing sites to proteolytic cleavage. At moderate enzyme concentrations, the 48-kDa fragment was protected by poly(G) against tryptic digestion. At the highest enzyme contentrations, both enzymes cleaved near the boundaries between repeats 2, 3, and 4 with some sites protected by poly(G), suggesting that the repeats themselves form compact units. The carboxyl-terminal domain was resistant to trypsin but was cleaved by chymotrypsin either in the presence or in the absence of poly(G), indicating exposure of some phenylalanines in this region. These studies provide a general picture of the topology of nucleolin and suggest that the nucleic acid binding region communicates with the amino-terminal domain.

Nucleolin, also called C23, is now the most thoroughly characterized protein from the nucleolus. Nucleolin has been implicated in the early stages of ribosome assembly because of its location in the dense fibrillar component (Spector et al., 1984; Escande et al., 1985) and its association with rapidly labeled preribosomal RNA (Herrera & Olson, 1986). A fraction of nucleolin may also be associated with chromatin (Olson & Thompson, 1983) and possibly be involved in regulating the transcription of preribosomal RNA (Bouche et al., 1984; Egyhazi et al., 1988; Erard et al., 1988). In addition, Borer et al. (1989) have proposed that nucleolin is a carrier of ribosomal proteins into the nucleolus.

The primary structure is now known completely for nucleolin from hamster (Lapeyre et al., 1986, 1987) and mouse (Bourbon et al., 1988) and partially for rat (Rao et al., 1982; Mamrack et al., 1979), *Xenopus laevis* (Caizergues-Ferrer et al., 1989), and chicken (Borer et al., 1989). These studies suggested that the protein may be divided into three putative domains, each having unique structural and functional properties: an amino-terminal domain of about 300 residues, consisting of four highly acidic sequences interspersed among basic segments, a central domain composed of four closely related 90-residue repeats, and a carboxyl-terminal domain (approximately 60 residues), which is enriched in glycine but contains phenylalanine and dimethylarginine.

It was shown by Bugler et al. (1987) that CNBr fragments from the central domain are capable of binding 18S or 28S ribosomal RNA. Furthermore, Sapp et al. (1989) isolated a proteolytic 48-kDa fragment of nucleolin that binds nucleic acids and contains only the central and carboxyl-terminal domains. The latter study also indicated that the 48-kDa fragment is more globular than the parent molecule. These data suggest that nucleolin has a central globular region that binds nucleic acids and an amino-terminal domain involved in protein–protein interactions or in modulating nucleic acid binding activity.

The apparent domain structure coupled with previous success using limited proteolysis pointed to the feasibility of this approach to further map the topology of nucleolin. It was found that the amino-terminal portion of nucleolin is highly susceptible to proteolysis, which is enhanced by the presence of nucleic acid. Furthermore, certain cleavage sites in the C-terminal half of the molecule are protected by nucleic acid.

Experimental Procedures

Purification of Nucleolin. Nucleoli were prepared from Novikoff hepatoma as previously described (Rothblum et al., 1977). A fraction enriched in nucleolin was obtained by extraction of nucleoli with low ionic strength buffers (Herrera & Olson, 1986). The extracts were stored at −80 °C in a buffer containing 10 mM tris-HCl (pH 7.4), 0.5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF),[1] 1.5 μmol/mL *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 10 μg/mL leupeptin, 1 μM aprotinin, 1 μM pepstatin, and 30% glycerol prior to use. Nucleolin was purified by chromatography on heparin–Sepharose by a procedure similar to a

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[1] Abbreviations: RNP, ribonucleoprotein; PMSF, phenylmethanesulfonyl fluoride; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; TPCK, *N*-tosylphenylalanine chloromethyl ketone; TLCK, *N*-tosyllysine chloromethyl ketone; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidine difluoride); PTH, phenylthiohydantoin.

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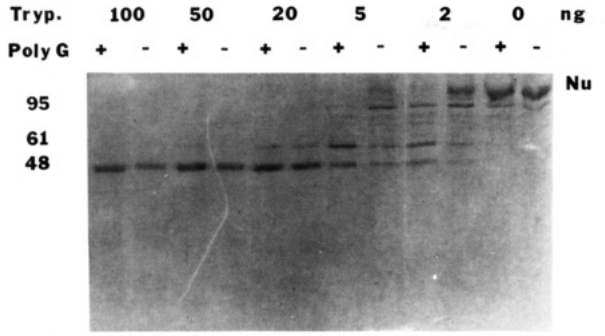

FIGURE 1: Effects of poly(G) on the limited proteolysis of the amino-terminal domain of nucleolin by trypsin. Samples of nucleolin (approximately 10 μg) were digested at 0–4 °C for 1 h with various amounts of trypsin (given in nanograms at top of figure) in the presence (+) or absence (−) of poly(G). The digestion products were subjected to SDS–PAGE and stained with Coomassie blue dye. The approximate molecular masses (in kdaltons) of the major fragments are indicated on the left side of the figure. The position of nucleolin (Nu) is indicated on the right.

previously described method (Caizergues-Ferrer et al., 1987).

Partial Proteolysis of Nucleolin. All manipulations of protein before and during digestion were carried out at 0–4 °C. Before digestion the column fractions containing purified nucleolin were dialyzed against 1 L of H1 buffer (50 mM tris-HCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.9) for 3–4 h. Aliquots containing 10–20 μg of nucleolin in 200 μL of H1 buffer were incubated for 10 min in the presence or absence of poly(G) (from Sigma) at a ratio of 200:1 (moles of nucleotides/mole of protein). Varying amounts of trypsin (Sigma, TPCK-treated) or α-chymotrypsin (Sigma, TLCK-treated) were added and digestion was continued for 1 h. The digestions were stopped by addition of TCA to a final concentration of 10% and washed by the procedure of Valenzuela et al. (1976). The washed precipitates were dissolved in SDS sample buffer and analyzed by electrophoresis on Laemmli-type gels (Laemmli, 1970) containing 12.5 or 15% polyacrylamide (SDS–PAGE).

Protein Sequencing. Proteolytic fragments separated by SDS–PAGE were transferred to PVDF membranes and stained with Coomassie blue essentially as described by Matsudaira (1987). Stained bands were placed directly in the sequencer cartridge between two cartridge seals. Protein sequencing was performed on an Applied Biosystems 470A sequencer essentially as previously described (Caizergues-Ferrer et al., 1989).

Results

Limited Proteolysis in the Amino-Terminal Domain of Nucleolin by Trypsin. To determine the effects of bound nucleic acids on the pattern of cleavage, limited proteolysis was performed with various concentrations of trypsin using paired samples of nucleolin where poly(G) was added to one of the samples in the pair. When the cleavage products were analyzed by SDS–PAGE (Figure 1), the previously observed 48-kDa polypeptide (Sapp et al., 1989) appeared as the major fragment when moderate amounts of trypsin (20–100 ng) were added to the digestion mixtures. At lower levels of trypsin (2 and 5 ng), two higher molecular weight fragments of 61 and 95 kDa were seen. These were presumably intermediates on the way to production of the 48-kDa fragment. In the latter samples, greater amounts of the parent protein and the 95-kDa fragment were seen in the absence of poly(G) and greater amounts of the 61- and 48-kDa fragments were observed in the presence of the nucleic acid. In other words, poly(G) appeared to enhance the breakdown of nucleolin. In the samples digested with 20–100 ng of trypsin, the 48-kDa band was more intense in the aliquots containing poly(G) than in the samples without nucleic acid. Thus, poly(G) protected the 48-kDa fragment from further degradation.

Separate digests were run on SDS–PAGE gels and transferred to PVDF membranes, and selected stained bands were run on the sequencer. The fragments were located in the polypeptide chain by comparison of the sequencing results to the known sequences of hamster (Lapeyre et al., 1987) and mouse (Bourbon et al., 1988) nucleolin using the residue numbers from the mouse sequence. The major site of cleavage to produce the 48-kDa fragment was after lysine 298. However, in experiments using low concentrations of trypsin, cleavage was also seen at lysines 283 and 295 (not shown), indicating that the 48-kDa fragment did not have a completely homogeneous amino terminus. These sites of cleavage are in the last basic region of the N-terminal third of the molecule and immediately upstream from the RNA binding region (Figure 2). Sequence analysis of the 61-kDa fragment indicated that the major cleavage site was at lysine 236, with

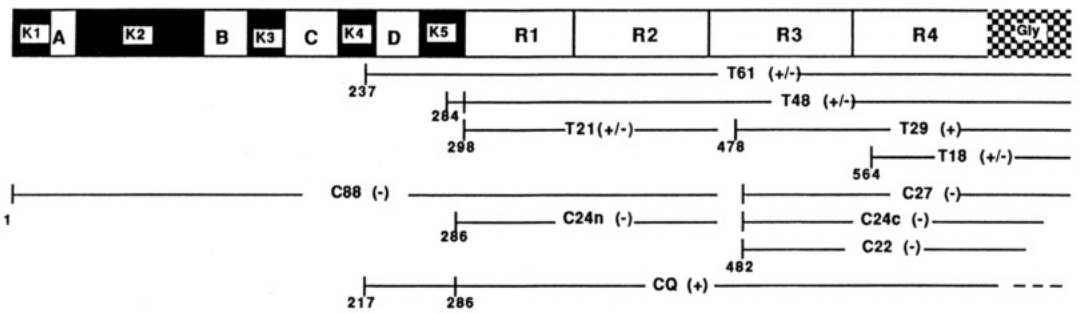

FIGURE 2: Location of proteolytic fragments in the nucleolin sequence. The upper part of the figure illustrates the unique segments found in the polypeptide chain. Darkly shaded areas (K1–K5) are lysine-rich regions, which are interspersed with acidic segments (clear areas A, B, C, and D). Clear areas with R1, R2, R3, and R4 represent the 80–90-residue repeated sequences. The fragments from limited proteolytic digests were isolated as described under Experimental Procedures and subjected to partial sequence analyses. The fragments were placed in the polypeptide chain by matching their amino-terminal sequences with the known sequence of mouse nucleolin. The fragments are identified by enzyme used (T for trypsin and C for chymotrypsin) followed by the approximate molecular mass in kdaltons. The 24-kDa chymotryptic fragments migrated as a single band containing two sequences: the two polypeptides are arbitrarily designated 24n and 24c. The line designated CQ represents a mixture of fragments ranging from 43 to 51 kDa containing two amino-terminal sequences and two or more carboxyl-terminal ends. The number under the vertical line at the amino-terminal end of each fragment indicates the residue number in mouse nucleolin where the fragment starts. (+) indicates that the fragment was produced only in the presence of poly(G), (−) indicates production of the fragment only in the absence of poly(G), and (+/−) indicates the fragment was produced either in the presence or in the absence of poly(G). The carboxyl-terminal ends of the fragments could not be precisely defined, as indicated by the absence of a vertical line or the presence of a dashed line at the right-hand side.

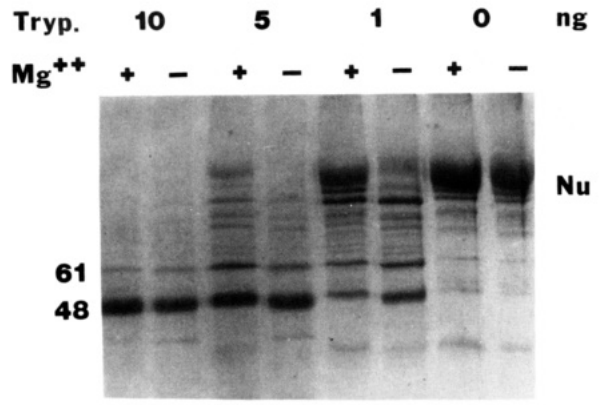

FIGURE 3: Effects of Mg^{2+} on the limited proteolysis of the amino-terminal domain of nucleolin by trypsin. Samples of nucleolin were digested with trypsin (given in nanograms at top of figure) as in Figure 1 in the presence of 5 mM $MgCl_2$ (+) or in the absence of metal ions but with 5 mM EDTA (–) in the digestion mixture. The approximate molecular masses (in kdaltons) of the major fragments are indicated on the left side of the figure. The position of nucleolin (Nu) is indicated on the right.

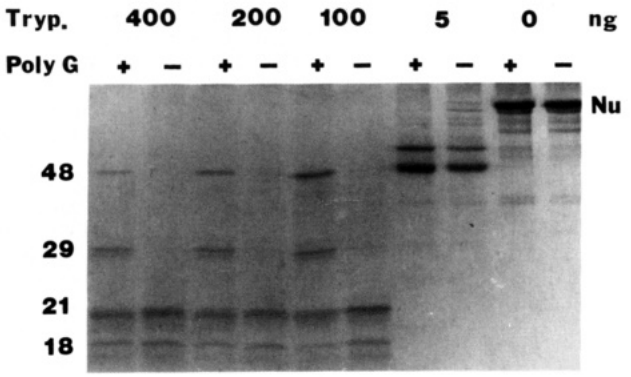

FIGURE 4: Effects of poly(G) on the limited proteolysis of the central domain of nucleolin by trypsin. Samples of nucleolin were digested as in Figure 1, except with higher amounts of trypsin (given in nanograms at top of figure) in the presence (+) or absence (–) of poly(G). The approximate molecular masses (in kdaltons) of the major fragments are indicated on the left side of the figure. The position of nucleolin (Nu) is indicated on the right.

secondary cleavages at lysines 225, 229, and 234. The latter group of cleavage sites are in the fourth basic region (K4), which separates two acidic segments (Figure 2). Thus, the sites of cleavage enhanced by the presence of poly(G) are in the two basic segments immediately upstream from the RNA binding region.

Studies by Sapp et al. (1986, 1989) suggest that the amino-terminal end of nucleolin participates in protein–protein interactions that are mediated through divalent metal ions. To further test this, paired samples of nucleolin were digested with various concentrations of trypsin with either 5 mM $MgCl_2$ or 5 mM EDTA present. Figure 3 shows that samples in which the divalent metal ions were removed were digested more rapidly than samples that contained $MgCl_2$. Thus, the amino-terminal domain is stabilized by this divalent metal.

Cleavage of the Central Domain of Nucleolin by Trypsin. To determine the location of protected regions, additional experiments were conducted at higher levels of trypsin (100–400 ng). In the absence of nucleic acid the stable digestion products were polypeptides of 18 and 21 kDa (Figure 4). The 21-kDa fragment was also seen in the presence of poly(G) at 100 ng of trypsin but the 18-kDa fragment was greatly reduced and replaced by a 29-kDa polypeptide. However, as trypsin concentrations were further increased in the poly(G)+ samples, the 18-kDa fragment appeared with

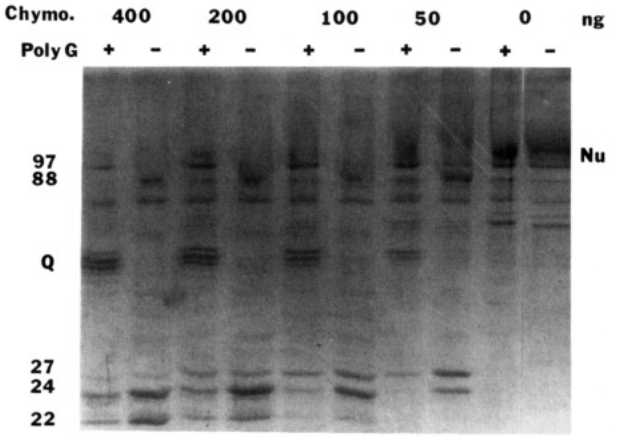

FIGURE 5: Effects of poly(G) on the limited proteolysis of the amino-terminal domain of nucleolin by chymotrypsin. Samples of nucleolin were digested as in Figure 1 with various amounts of chymotrypsin (given in nanograms at top of figure) in the presence (+) or absence (–) of poly(G). The approximate molecular masses (in kdaltons) of the major fragments are indicated on the left side of the figure. Q represents a group of four bands (quadruplets) having molecular masses of approximately 43, 45, 48, and 51 kDa. The position of nucleolin (Nu) is indicated on the right.

concomitant diminution of the 29-kDa fragment.

The 21-kDa fragment began at residue 299, which is the same location as the predominant amino terminus of the 48-kDa tryptic fragment (Figure 2). The 29-kDa fragment began at residue 478, indicating that cleavage at lysine 477 essentially split the RNA binding region into two halves by cutting the third 90-residue repeat (R3) near its interface with the second repeat (Figure 2). However, the 18-kDa fragment, which was produced in the absence of nucleic acid, began at residue 564. This site of cleavage is in repeat 4 near its interface with repeat 3, resulting in a stable fragment containing one of the putative RNA binding segments and the C-terminal glycine-rich segment (Figure 2). In the absence of poly(G), no stable fragments derived from repeat 3 were detected. This suggests that poly(G) protects a region surrounding arginine 563. Furthermore, the production of a limited number of subfragments from the 48-kDa fragment, even in the absence of bound nucleic acid, strongly implied that only a few of the large number of tryptic sites were exposed on the surface of the molecule.

Partial Proteolysis with Chymotrypsin. Similar experiments were done using chymotrypsin instead of trypsin as the digesting enzyme. In the absence of nucleic acid, chymotrypsin at all concentrations produced a major high molecular weight polypeptide of approximately 88 kDa (Figure 5). This was accompanied by the appearance of two smaller fragments (24 and 27 kDa) at low enzyme concentrations. At higher enzyme concentrations the 27-kDa band was diminished, concurrent with the appearance of a 22-kDa fragment. A 62-kDa fragment also began to appear at higher enzyme concentrations (see below). Sequence analysis indicated that the 88-kDa fragment had the same amino-terminal sequence as the parent protein, placing it at the amino-terminal end. The 22- and 27-kDa fragments had the same N-terminal sequences, which began at residue 482. The 24-kDa band had similar amounts of two sequences, beginning at residues 286 and 482. This indicates that the 24-kDa band contains two polypeptides resulting from cleavage at methionine 285 and tryptophan 481. Because these two polypeptides could not be separated from each other by electrophoretic methods, they were designated 24n and 24c (Figure 2). The cleavage sites to produce the amino-terminal ends of this set of fragments are strikingly close

to major sites of tryptic cleavage (residues 299 and 478) to produce the 48- and 29-kDa fragment, respectively. Thus, residues in these regions, which are near the interfaces between the amino-terminal and central domains and between repeats 2 and 3 (Figure 2), appear to be exposed for cleavage by chymotrypsin as well as by trypsin.

Since the 22- and 27-kDa fragments and one component of the 24-kDa band (24c) all had the same amino-terminal sequence, further cleavage must be occurring near the carboxyl-terminal end of the molecule; i.e., the two shorter fragments are derived from the 27-kDa fragment. This suggests that the 90-residue repeats (R3 and R4, Figure 2) are relatively stable, compact units and the carboxyl-terminal glycine-rich region, which contains seven residues of phenylalanine, is exposed for digestion by chymotrypsin. However, it could not be determined at which phenylalanine residues cleavage was occurring.

In the presence of poly(G) the 88-, 27-, 24-, and 22-kDa fragments were reduced in amounts at all enzyme concentrations (Figure 5). Since all of these fragments except the 24n component are generated by cleavage at tryptophan 481, the latter residue must be protected by the presence of nucleic acid. This residue is only four positions downstream from lysine 477, which is not protected by poly(G) (Figure 2; see above), suggesting that residues in the vicinity of these cleavage sites are at the boundaries of the nucleic acid binding regions.

A new set of digestion products was seen in the presence of poly(G): a 97-kDa fragment and a set of four or more closely spaced bands estimated to have molecular weights of 43, 45, 48, and 51 kDa (Figure 5). These fragments (referred to as the quads) were sequenced as a single band. Two predominant sequences were obtained, one beginning at glutamic acid 217 and the other at threonine 286 (Figure 2). These sites are in basic segments 4 and 5 (Figure 2), which also show enhanced tryptic cleavage in the presence of nucleic acid (see above). This further supports the idea that these two segments are generally susceptible to cleavage by more than one enzyme. The multiplicity of fragments produced by chymotrypsin would appear to result from multiple cleavage sites in the C-terminal end of the molecule, as seen with the 22-, 24-, and 27-kDa chymotryptic fragments produced in the absence of nucleic acid.

Another interesting observation is that the 88-kDa fragment, which contains the amino-terminal domain, remained relatively intact at all enzyme concentrations in the absence of nucleic acid. However, the 88-kDa polypeptide was markedly diminished in digestion mixtures where poly(G) was added, with concurrent appearance of the quad bands. Since the quad bands arise from cleavage in the basic K4 and K5 segments of the amino-terminal domain, the presence of nucleic acid must induce alterations that affect chymotryptic as well as tryptic sites in these segments.

Discussion

Nucleolin contains two segments that are highly susceptible to cleavage by the two enzymes tested. These are basic segments 4 and 5, which are separated by acidic segment D and immediately precede the nucleic acid binding region (see Figure 6). The sensitivity of these segments may be due to a combination of factors. First, the hydrodynamic studies of Sapp et al. (1989) suggest that the amino-terminal third of the molecule is less compact than the C-terminal two-thirds, thereby increasing the overall susceptibility of that part of the polypeptide. Second, both of these segments are rich in lysine but they also contain alanine, valine, threonine, and proline. The latter amino acid would be expected to produce kinks in

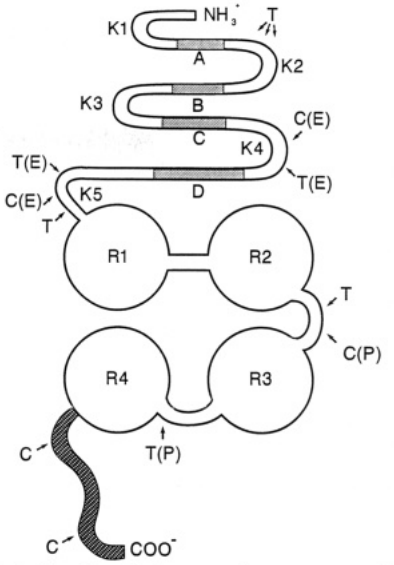

FIGURE 6: Relationship of cleavage sites to proposed general conformation of nucleolin. The highly schematic model of the conformation is based on secondary structure predictions, hydrodynamic studies, and the results of partial proteolysis experiments (see text). The various segments correspond to those illustrated in Figure 2. The amino-terminal one-third is composed of highly acidic regions (A–D) interspersed between lysine-rich segments that also contain proline (K1–K5). This highly polar amino-terminal domain appears to be responsible for the elongated shape of the molecule. The central domain is proposed to be a relatively compact unit consisting of four 80–90-residue repeats (R1–R4) represented by circles. Repeats R2, R3, and R4 appear to be connected by relatively labile segments. The cross-hatched segment represents the carboxyl-terminal domain, which is enriched in glycine and dimethylarginine and is also predicted to be of a nonglobular conformation. The cleavage sites at their approximate locations are designated by arrows with letters representing the cleaving enzyme (T for trypsin and C for chymotrypsin). The letters in parentheses indicate whether cleavage at that site was protected (P) or enhanced (E) by the presence of nucleic acid.

the polypeptide chain and prevent formation of regular secondary structures. Secondary structure predictions (Chou & Fasman, 1978; Rose, 1978; Garnier et al., 1978) suggest that the cleavage sites are either in or near regions containing predominantly random coil conformations, which should be more susceptible to proteolysis than ordered structures. Finally, the segments cleaved are not only moderately hydrophilic themselves, but they are also adjacent to the highly acidic segments (Figures 2 and 6), which may draw the cleavage sites to the surface of the molecule, thereby enhancing their susceptibility to proteases.

Several nucleic acid binding proteins exhibit the property of increased susceptibility to proteolysis upon binding nucleic acids. These include the T4 gene 32 protein (Williams & Konigsberg, 1978), the *Escherichia coli* SSB protein (Williams et al., 1983) and the *Xenopus* transcription factor IIIA (Hanas et al., 1989). In each of these cases the increased sensitivity has been attributed to a change in the conformation of the protein when nucleic acid is bound. This would also appear to be the case with nucleolin since the enhanced cleavage sites are relatively close to the nucleic acid binding region. It is conceivable that conformational changes are a means by which the nucleic acid binding region of the molecule communicates with the amino-terminal end. This could serve to modulate protein–protein interactions or regulate parts of the ribosome assembly process.

In the nucleic acid binding region of nucleolin, proteolysis by trypsin and chymotrypsin was limited to sites near the boundaries between repeats 2 and 3 and repeats 3 and 4 (Figure 2). The cleavage sites are contained in hydrophilic

regions (Bugler et al., 1987), which are predicted either to have a high β-turn potential or to be essentially devoid of secondary structure. These would appear to serve as linkers between more compact units containing the individual repeats (see Figure 6). Each of these repeats potentially contains a nucleic acid binding site, since one of them alone is capable of binding RNA (Bugler et al., 1987). These repeats may serve as separate globular units.

The carboxyl-terminal end of nucleolin was susceptible only to chymotrypsin, with no evidence of protection by nucleic acids. Although it was not possible to pinpoint the sites of cleavage, the phenylalanine residues are the most likely candidates. We also speculate that trypsin does not cleave in this region because dimethylarginine is a poor substrate for that enzyme. Although there is presently no function ascribed to this part of the molecule, the current work suggests that it is relatively exposed and free to interact with the solvent or other nucleolar macromolecules.

Acknowledgments

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Supplementary Material Available

Data from Edman degradation of isolated tryptic and chymotryptic fragments of nucleolin (4 pages). Ordering information is given on any current masthead page.

Registry No. Poly(G), 25191-14-4.

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