DO ANTIBODY BINDING TECHNIQUES IDENTIFY POLYSOMES SYNTHESIZING A SPECIFIC PROTEIN?

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SUMMARY

Specific binding of antibody directed against MOPC-21 myeloma protein to MOPC-21 polysomes has been demonstrated. It was also shown that this same antibody bound specifically to the ribosomal subunits of these polysomes, suggesting that the antibody is not binding exclusively to the nascent polypeptide chains on the polysomes. The antibody was bound specifically to polysomes of a nonproducing variant XC1 which had been isolated in the presence of MOPC-21 myeloma protein while the antibody was not bound to XC1 polysomes isolated in the absence of myeloma protein. This suggests that the myeloma protein is adsorbed to the polysomes during the isolation procedure.

INTRODUCTION

The use of specific antibody to localize and isolate polyribosomes synthesizing a particular protein has been reported for a variety of proteins. Among those proteins studied have been rat serum albumin (1-4), ovalbumin (5), β-galactosidase (6,7), triose phosphate dehydrogenase (8), glutamic dehydrogenase (9), glutamine synthetase (10), catalase (11), thyroglobulin (12), collagen (13), hemoglobin (14), immunoglobulin light chains (15-17), and complete immunoglobulin molecules (4,16,18-21). In principle, antibody directed against a specific protein binds only to those nascent polypeptide chains on polysomes synthesizing that protein. This method has been used to determine the size and relative number of specific polysomes as well as to isolate the specific

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Abbreviations: NRGG, normal rabbit gamma globulin; EDTA, ethylenediaminetetraacetic acid.

polysomes as an initial step in the purification of a specific messenger RNA.

Our work has been done with the murine myeloma cell line MOPC-21 which produces and secretes a gamma globulin molecule of the IgG-1 type. We report here evidence suggesting that intracellular gamma globulin (myeloma protein) is being adsorbed onto the ribosomes during polysome isolation. Thus, the specific antiserum (anti-globulin) is not binding exclusively to the myeloma protein nascent chains.

MATERIALS AND METHODS

Cells and Tumors: The myeloma tumor MOPC-21 was maintained in tissue culture and in female BALB/c mice as an ascites tumor as previously described (22). The myeloma cell line XC1 was maintained in the same manner as the MOPC-21 cell line in tissue culture only.

Proteins: The MOPC-21 myeloma protein (IgG-1) was isolated from the ascites fluid of tumor-bearing mice. The protein was precipitated twice with saturated ammonium sulfate [final concentration 40% (v/v)]. The precipitate was redissolved and dialyzed against 5 mM sodium phosphate, pH 8.0. The protein was then applied to a DEAE-cellulose column (Sigma) equilibrated in the same buffer and eluted with a linear gradient of 5-100 mM sodium phosphate, pH 8.0. The first peak eluted contained the gamma globulin.

Antiserum against the myeloma protein was raised in New Zealand White rabbits by injecting subcutaneously 5 mg of myeloma protein in Freund's complete adjuvant. The animals were periodically given booster injections and bled through the marginal ear vein 7 to 10 days after each injection. The gamma globulin fraction of the serum from immunized rabbits (anti-globulin) and non-immunized rabbits (NRGG) was purified as described for the myeloma protein. The rabbit anti-myeloma protein (anti-globulin) was then purified by affinity chromatography on Sepharose-4B coupled with myeloma protein. The antibody was eluted with 4.5 M $MgCl_2$ as described by Shapiro et al. (23).

Initially in these studies purified anti-globulin labeled with ^{14}C was used. This *in vitro* labeling was done by the procedure of Rice and Means (24). Typical specific activities were 8,000 to 12,000 cpm/µg protein. Purified rabbit anti-globulin was also labeled with ^{125}I using a modification of the chloramine-T procedure reported by Sonoda and Schlamowitz (25). Potassium iodide (10 µg/ml), chloramine-T (1 mg/ml) and sodium metabisulfite (1 mg/ml) were dissolved in 0.2 M sodium phosphate (pH 7.0). To 100 µg purified anti-globulin was added 0.125 µg KI followed by 50 µCi Na ^{125}I (New England Nuclear). The solution was neutralized with 0.1 N HCl, 4 µg of chloramine-T was added and the reaction volume was brought to 0.1 ml with 0.2 M sodium phosphate (pH 7.0). The reaction vessel was tightly capped and incubated with stirring for 10 minutes on ice. The reaction was then stopped by the addition of 4 µg sodium metabisulfite and 0.2 M sodium phosphate was added to a final volume of 1.0 ml. The solution was then dialyzed overnight against 10 mM Tris (pH 7.4), 150 mM NaCl to remove any free iodine. Typical specific activities were around 100,000 cpm/µg protein.

Isolation of Polysomes: Polysomes were isolated from both tissue culture

cells and ascites tumor cells as previously described (22). Briefly, cells were lysed by the addition of Nonidet P-40 (Shell) at a final concentration of 0.5% (v/v). After removal of the nuclei by centrifugation, the supernatant was treated with sodium deoxycholate and Triton X-100 at a final concentration of 0.5% (w/v) and 0.5% (v/v), respectively. The solution was then applied to a Sepharose-6B column and the purified polysomes eluted with the void volume. Ribosomal subunits were prepared by adding EDTA, pH 7.0, to the purified polysome solution at a final concentration of 33 mM and incubating for 10 minutes on ice.

Binding of Antibody to Polysomes: Direct binding studies were done by adding labeled anti-globulin to polysome solutions (approximately 1.25 µg antibody per A_{260} unit polysomes) and incubating 1 hour on ice. For the inhibition studies, unlabeled competing protein (anti-globulin or NRGG) at 60 to 65 µg per A_{260} unit polysomes was added and the solution incubated on ice for 30 minutes. Labeled anti-globulin was then added and the incubation continued for another 30 minutes.

The polysomes were analyzed on 15 to 45% (w/v) linear sucrose gradients (in polysome buffer: 25 mM Tris, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$, 100 µg/ml heparin). The gradients were centrifuged at 45,000 rpm (250,000 x g) for 35 minutes at 4° C in a Beckman SW50.1 rotor in a Beckman model L5-50 ultracentrifuge. Subunits were centrifuged through 10 to 30% (w/v) linear sucrose gradients (in 10 mM Tris, pH 7.4, 10 mM NaCl, 10 mM EDTA) for 170 minutes at 45,000 rpm in the SW50.1 rotor. Fractions of 0.2 ml were collected from the top using an ISCO density gradient fractionator. Absorbance at 254 nm was monitored continuously with the ISCO model UA-4 absorbance monitor and

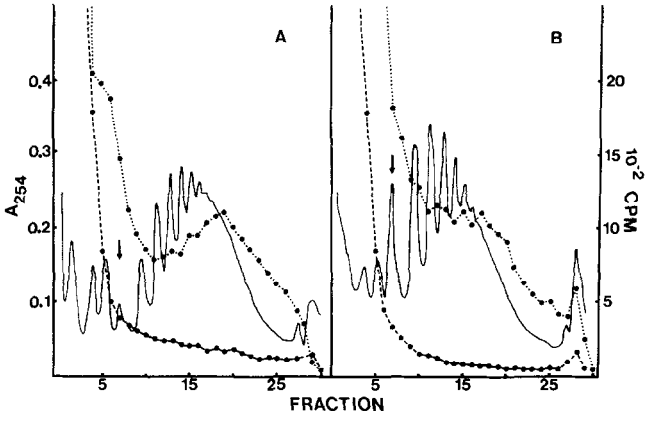

FIGURE 1. MOPC-21 polysomes (2.5 A_{260}) were incubated with ^{125}I-anti-globulin (72,000 cpm/µg), centrifuged on 15-45% linear sucrose gradients and 0.2 ml fractions collected as described in Materials and Methods. Arrow indicates position of monosomes. A_{254}(———). A. Polysomes incubated with ^{125}I-anti-globulin (●·····●) or pre-incubated with unlabeled anti-globulin followed by incubation with ^{125}I-anti-globulin (●---●). B. Polysomes pre-incubated with unlabeled NRGG followed by incubation with ^{125}I-anti-globulin (●·····●) or ^{125}I-anti-globulin centrifuged on a control gradient without polysomes (●---●).

recorded on a Gilford recorder. Fractions containing iodinated protein were counted in a Packard gamma scintillation counter. Fractions containing ^{14}C-labeled protein were added to 5 ml Bray's scintillation fluid (25) and counted in a Packard Tri-Carb liquid scintillation counter.

RESULTS

Iodinated anti-globulin was incubated with MOPC-21 polysomes and analyzed on sucrose gradients as described in Materials and Methods. Fig. 1A shows a typical profile of labeled antibody (dotted line) incubated with the polysomes. Antibody co-sediments with the polysomes throughout the gradient with a broad peak in the region of the largest polysomes. The dashed line indicates the sedimentation profile of labeled antibody added to polysomes pre-incubated with unlabeled anti-globulin. The binding of the labeled antibody has been reduced in the polysome region of the gradient as well as in the region of the monosomes (arrow) and subunits. Of the labeled anti-globulin added, 3.2% sedimented in the subunits-monosome region (fractions 4-8) and 3.2% in the polysome region (fractions 9-30) compared with 6.0% and 11.6%, respectively, for binding of labeled anti-globulin in the absence of unlabeled competing anti-globulin. Fig. 1B shows binding of antibody to polysomes pre-incubated with unlabeled NRGG (dotted line) and the sedimentation of labeled antibody in a control gradient containing no polysomes (dashed line). There was no significant inhibition of binding by NRGG (9.8% in the subunits-monosome region and 12.2% in the polysome region) while the results of the blank gradient were similar to those seen with inhibition by unlabeled anti-globulin (3.0% in the subunits-monosome region and 1.9% in the polysome region). Table I summarizes the results of several other binding experiments that were done using ^{14}C-labeled anti-globulin. Again, unlabeled specific antibody reduced the amount of binding of specific antibody while unlabeled NRGG had no inhibitory effect. The fact that the binding of labeled anti-globulin to MOPC-21 polysomes is almost completely inhibited by excess unlabeled anti-globulin but is unaffected by similar amounts of unlabeled nonspecific gamma globulin indicates that the binding is specific for myeloma protein determi-

TABLE I

BINDING OF ^{14}C-ANTI-GLOBULIN TO SEPHAROSE PURIFIED POLYSOMES

Experiment	Source of Polysomes	Unlabeled Competing Protein	Percent CPM Binding To Subunits-Monosomes[a]	Percent CPM Binding To Polysomes[a]
1	MOPC-21	None	26.2	17.9
	MOPC-21	Anti-globulin	3.2	2.8
2	MOPC-21	None	47.1	26.5
	MOPC-21	Anti-globulin	16.8	7.9
3	MOPC-21	None	22.8	17.0
	MOPC-21	None	27.6	16.8
	MOPC-21	None	24.6	22.4
	XC1	None	6.4	6.9
4	MOPC-21	Anti-globulin	7.7	2.6
	MOPC-21	NRGG	15.8	11.8

[a]Percent CPM binding to subunits-monosomes and polysomes calculated from radioactivity recovered from gradient (Does not take into account the 35-50% that pelleted).

nants on the polysomes. In experiment 3 binding was attempted with polysomes isolated from XC1 cells. This line is a synthesis variant of the myeloma cell line C1 which no longer produces myeloma protein (27). As would be expected, there is little binding of labeled anti-globulin to polysomes from these cells. (See also Fig. 3.)

It should be noted that in all binding experiments which used ^{125}I-antibody,

a large amount of the antibody pelleted through the sucrose gradients. The amount ranged from 35 to 50% of the total radioactivity added. In all experiments using ^{125}I-antibody the pelleted radioactivity was added to the total radioactivity in the gradient for the purpose of determining the percentages in each region. Some of the pelleted radioactivity may be due to cross-linking of polysomes by the antibody, forming aggregates large enough to sediment to the bottom of the tube. However, even on control gradients containing no polysomes, as much as 35 to 40% of the added radioactivity pelleted. This may be the result of the interaction of antibody molecules forming large aggregates, a phenomenon that has been noted by other workers (13).

As mentioned earlier, there appeared to be some specific binding of anti-globulin to monosomes and subunits as well as to polysomes. To investigate

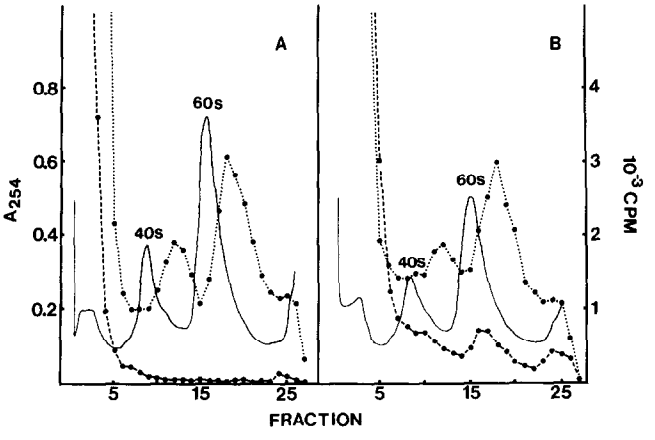

FIGURE 2. MOPC-21 polysomes were dissociated by addition of EDTA (33mM final concentration), incubated with ^{125}I-anti-globulin (79,000 cpm/µg) and centrifuged on 10-30% linear sucrose gradients as described in Materials and Methods The 40 S and 60 S notations are intended only to indicate the positions of the small and large ribosomal subunits and do not necessarily correspond to the actual S values. A_{254}(———). A. Subunits (2.1 A_{260}) incubated with ^{125}I-anti-globulin (●·····●) or ^{125}I-anti-globulin centrifuged on a control gradient without subunits (●---●). B. Subunits (2.1 A_{260}) pre-incubated with unlabeled NRGG (●·····●) or unlabeled anti-globulin (●---●) followed by incubation with ^{125}I-anti-globulin.

this, MOPC-21 polysomes were dissociated by treating with EDTA and then incubated with ^{125}I-anti-globulin. The results are shown in Fig. 2. Fig. 2A shows the absorbance profile of the small and large subunits (solid line) along with the sedimentation pattern of the labeled anti-globulin which has been incubated with the subunits (dotted line). Also shown is the sedimentation profile of ^{125}I-anti-globulin centrifuged on a control gradient (dashed line). Of the anti-globulin incubated with the subunits, 31.1% sedimented with the subunits (fractions 7-26) while on the control gradient only 2.2% of the antibody was found in this region. The specificity of this binding is shown in Fig. 2B. The subunits were pre-incubated with either unlabeled anti-globulin (dashed line) or unlabeled NRGG (dotted line) before addition of ^{125}I-anti-globulin. The NRGG had little effect (30.6% of the antibody co-sedimented with the subunits) while the unlabeled anti-globulin substantially reduced the binding (11.3%). The double peaks of antibody indicate that the antibody is binding to both the small and large subunits. Since dissociation of the polysomes into subunits should release the nascent chains, one would not expect to find specific binding of antibody to individual subunits. The results shown in Fig. 2, then,

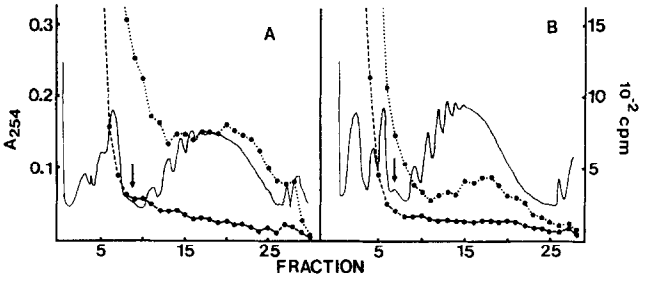

FIGURE 3. XCl polysomes (1.8 A_{260}) were incubated with ^{125}I-anti-globulin and centrifuged on 15-45% linear sucrose gradients as described in Materials and Methods. Arrow indicates position of monosomes. A_{254}(———). A. XCl polysomes isolated in the presence (●·····●) or absence (●---●) of myeloma protein and incubated with ^{125}I-anti-globulin (78,000 cpm/µg). B. XCl polysomes isolated in the presence of myeloma protein, pre-incubated with unlabeled NRGG (●·····●) or unlabeled anti-globulin (●---●) followed by incubation with ^{125}I-anti-globulin (62,000 cpm/µg).

would suggest that specific antigen (nascent pepetide chains, completed myeloma molecules, or both) must be bound to the ribosomal subunits.

To test if intracellular myeloma protein could bind to ribosomes during the isolation procedure the following experiment was done using the nonproducing XC1 cells. The total polysome fraction was isolated from XC1 cells by the same procedure as previously reported (22) with one exception. Immediately before lysing the cells with Nonidet P-40, MOPC-21 myeloma protein was added to the suspension at a final concentration of approximately 15 picograms per cell. This value was determined to be the approximate intracellular concentration of myeloma protein in exponentially growing MOPC-21 tissue culture cells (data not shown). The XC1 polysomes were then isolated and purified in the normal manner and binding studies with ^{125}I-anti-globulin were done. The results are shown in Fig. 3. In Fig. 3A the dashed line represents the sedimentation profile of ^{125}I-anti-globulin incubated with polysomes isolated normally from XC1 cells (i.e., not isolated in the presence of myeloma protein). The dotted line corresponds to the labeled anti-globulin incubated with XC1 polysomes isolated in the presence of myeloma protein. In the latter case, 13.0% of the anti-globulin sedimented in the subunits-monosome region (fractions 5-10) and 10.8% sedimented in the polysome region. In the former case, only 3.8% of the anti-globulin bound in the subunits-monosome region and 2.3% in the polysome region. The specificity of the binding is demonstrated in Fig. 3B. Pre-incubation with unlabeled NRGG (dotted line) had little effect on binding (13.1% in subunits-monosome region; 8.1% in polysome region), while pre-incubation with unlabeled anti-globulin significantly reduced the binding (2.4% in subunits-monosome region; 2.6% in the polysome region). Thus, ^{125}I-anti-globulin binds specifically to polysomes isolated in the presence of myeloma protein while it does not bind to normal XC1 polysomes, indicating that at least some of the myeloma protein added prior to polysome isolation must be bound to the XC1 ribosomes.

DISCUSSION

The results of direct binding of ^{125}I-anti-myeloma protein (anti-globulin) to polysomes isolated from MOPC-21 cells are consistent with specific binding of the anti-globulin to ribosomes. This binding has been presumed to be due to the interaction of the antibody with the nascent peptide chains on those polysomes synthesizing the myeloma protein. However, binding of the antibody to the monosomes and subunits in the polysomes preparation was noted. Incubation of labeled anti-globulin with EDTA-treated MOPC-21 polysomes showed significant specific binding to both the small and large subunits. Interestingly the peaks of bound antibody were always shifted to the heavy side of the absorbance profiles of the subunits, suggesting that most of the bound antibody is bound to only a small fraction of the subunits.

The results shown in Fig. 3 indicate that the myeloma protein binds to ribosomes during the polysome isolation procedure. Adsorption of membrane proteins to ribosomes during isolation of membrane-bound polysomes from rat liver cells has been reported (28). The proteins adsorbed were from solubilized endoplasmic reticulum and the amount of binding could be reduced by increasing the salt concentration or by using deoxycholate instead of Triton X-100 to solubilize the membranes. Moav and Harris (29) have reported the adsorption of immunoglobulin to rabbit lymph node polysomes. They found that the gamma globulin was bound through the F_c protion of the molecule. They were unable to completely remove the bound protein without dissociating the ribosomes.

Myeloma protein released when the rough endoplasmic reticulum is solubilized may be adsorbing to the ribosomes. Since we isolate the total polysome population (i.e., both free and membrane-bound) one would expect the adsorption, if completely random, to be uniform over the entire polysome population. As noted above, however, the results of binding specific antisera to MOPC-21 ribosomal subunits suggest that only a fraction of the total population is involved. We are currently investigating the binding of anti-globulin to free and membrane-bound polysomes and subunits isolated from MOPC-21 cells under

various conditions to try to determine if only a specific subpopulation of polysomes has adsorbed myeloma determinants.

Our results indicate that in the MOPC-21 system the binding of specific antiserum against the myeloma protein may not be identifying only those polysomes synthesizing the myeloma protein. When considered with other reports in the literature (28,29) these results suggest that adsorption of intracellular protein to ribosomes may be a problem, particularly in systems involving gamma globulin.

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