Probing the mRNA processing body using protein macroarrays and "autoantigenomics"

WEI-HONG YANG and DONALD B. BLOCH

Center for Immunology and Inflammatory Diseases of the General Medical Services, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02129, USA

ABSTRACT

Messenger RNA processing bodies (P-bodies) are cellular structures that have a direct role in mRNA degradation. P-bodies have also been implicated in RNAi-mediated post-transcriptional gene silencing. Despite the important roles of P-bodies in cellular biology, the constituents of P-bodies and their organization have been only partially defined. Approximately 5% of patients with the autoimmune disease primary biliary cirrhosis have antibodies directed against these structures. Recent advances in protein macroarray technology permit the simultaneous screening of thousands of proteins for reactivity with autoantibodies. We used serum from patients with anti-P-body autoantibodies to screen a protein macroarray and identified 67 potential autoantigens. Immunoreactive proteins included four known P-body components and three additional primary biliary cirrhosis autoantigens. Y-box protein 1 (YB-1), a 50-kDa RNA-binding protein that was not previously known to be a P-body component, was recognized by serum from four of seven patients. YB-1 colocalized with P-body components DCP1a and Ge-1. In cells subjected to arsenite-induced oxidative stress, YB-1 localized to TIA-containing stress granules. Both YB-1 and the previously identified P-body component RAP55 translocated from P-bodies to stress granules during oxidative stress. During recovery, however, the reappearance of YB-1 in P-bodies was delayed compared with that of RAP55, suggesting that YB-1 and RAP55 may have different functions. This study demonstrates that the combination of human autoantibodies and protein macroarray technology provides a novel method for identifying and characterizing components of mRNA P-bodies.

Keywords: mRNA processing body; stress granule; mRNA decay; autoantigen; protein macroarray

INTRODUCTION

Messenger RNA storage and degradation are critical steps in the regulation of gene expression. Cytoplasmic mRNA processing bodies (P-bodies, also known as "cytoplasmic foci" and GW182 bodies) are multiprotein complexes that have a central role in the regulation of mRNA metabolism (Eystathioy et al. 2003; Sheth and Parker 2003; Cougot et al. 2004). P-bodies contain decapping enzymes (DCP1a and DCP2) that remove the 5′ cap of mRNA and a 5′ to 3′ exoribonuclease (Xrn1). These structures also contain seven "Sm-like" proteins (LSm1–7), which form a heptameric complex that binds mRNA, and the DEAD box family helicase Rck/p54, which increases the efficiency of mRNA decapping (Bouveret et al. 2000; Coller et al. 2001; Eystathioy et al. 2003; Cougot et al. 2004). Three autoantigens, GW182, Ge-1, and RAP55, also localize to P-bodies (Eystathioy et al. 2003; Fenger-Gron et al. 2005; Yu et al. 2005; Yang et al. 2006). GW182 recruits Argonaute proteins, key components of the RNA interference machinery, to P-bodies (Jakymiw et al. 2005; Liu et al. 2005). Silencing of GW182 disrupts P-bodies and inhibits translational repression by miRNA, suggesting that there is a functional link between RNAi and P-bodies (Jakymiw et al. 2005; Liu et al. 2005). Ge-1, also known as Hedls, interacts with the core decapping enzymes and enhances mRNA degradation (Fenger-Gron et al. 2005; Yu et al. 2005). The third P-body autoantigen, RAP55, contains an Sm-like domain and two RGG-type RNA-binding motifs. RAP55 localizes to P-bodies and, in response to oxidative stress, translocates to stress granules. RAP55 rapidly returns to P-bodies during the recovery period, suggesting that RAP55 may have a role in shuttling mRNA between stress granules and P-bodies (Yang et al. 2006).

P-bodies have a dynamic relationship with polyribosomes and stress granules (Brengues et al. 2005; Kedersha et al. 2005; Yu et al. 2005). In cells that are treated with cyclohexamide, a translation elongation inhibitor that traps mRNAs in polysomes, P-bodies rapidly disappear. In contrast, displacement of mRNAs from polysomes by puromycin increases the size

Reprint requests to: Donald B. Bloch, Center for Immunology and Inflammatory Diseases of the General Medical Services, Massachusetts General Hospital-East, CNY 8302, 149 13th Street, Charlestown, MA 02129, USA; e-mail: bloch@helix.mgh.harvard.edu; fax: (617) 726-5651.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.411907.

and number of P-bodies. During environmental stress, translation of mRNAs encoding housekeeping proteins is discontinued, and these mRNAs are stored in stress granules for potential future use (for review, see Kedersha and Anderson 2002). Stress granules contain small ribosome subunits, translation initiation factors, and a variety of RNA-binding proteins. Antibodies directed against TIA, a central component of stress granules, serve as a marker for these structures. The sequestration of housekeeping mRNAs permits mRNAs encoding cellular repair proteins to gain access to the translation machinery. P-bodies and stress granules interact with each other during and after environmental stress, and some P-body components, including RAP55 and XrnI, are found in both P-bodies and stress granules (for review, see Anderson and Kedersha 2006). During the recovery period, stress granules may serve as a "sorting" domain for mRNAs. A subset of mRNAs may be recycled and returned to polysomes to restart protein synthesis. In contrast, other mRNAs may be transferred to adjacent P-bodies for destruction. The mechanism by which mRNAs are separated, for either reuse or destruction, is unknown.

Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology characterized by the progressive destruction of intrahepatic biliary ductules, leading to hepatic fibrosis and liver failure (for review, see Kaplan and Gershwin 2005). Approximately 5% of PBC patients have antibodies directed against P-bodies (Bloch et al. 2005). Recent advances in the field of proteomics have facilitated the development of a membrane-based macroarray that permits high-throughput screening of human proteins using autoantibodies (Bussow et al. 2000). The technique involves preparation of a cDNA library in a prokaryotic expression vector, transformation of bacteria, and growth of individual clones in microtiter wells. The bacteria are robotically transferred, at high density, to nitrocellulose membranes and are induced to express the cDNA in situ. This technique was used by other investigators to identify the targets of autoantibodies in patients with cardiac and dermatologic disorders (Lueking et al. 2005; Horn et al. 2006). In the present study, we used serum from patients with primary biliary cirrhosis and protein macroarray technology to identify potential P-body autoantigens and show that Y-box protein 1 (YB-1) is a novel component of these structures.

RESULTS AND DISCUSSION

PBC autoantibodies react with proteins in a macroarray

Using indirect immunofluorescence and the Hep-2 cell line as a substrate, 34 sera were found to contain antibodies that reacted with 5–20 discrete dots in the cell cytoplasm. Human antibodies colocalized with rabbit anti-DCP1a antibodies, confirming that these sera contained anti-P-body antibodies (Bloch et al. 2005; Yu et al. 2005; Yang et al. 2006). Seven of the 34 human sera were used in this study to screen a protein macroarray prepared from phytohemagglutinin-treated human T-lymphocytes. The macroarray consists of a membrane with a 48-by-48 pattern of blocks. Each of the blocks contains 25 squares and 12 proteins are present, in duplicate, surrounding a central ink spot (see Fig. 1). The array contains ~17,000 proteins. A total of 67 immunoreactive proteins were identified (Table 1). These proteins included four known P-body components, DCP1a, TTP, Ge-1, and RAP55 (van Dijk et al. 2002; Fenger-Gron et al. 2005; Yu et al. 2005; Anderson and Kedersha 2006; Yang et al. 2006). In addition to Ge-1 and RAP55, three other PBC autoantigens, E3 pyruvate dehydrogenase complex, lamin A/C, and valosin-containing protein, were identified (Courvalin and Worman 1997; Palmer et al. 1999; Miyachi et al. 2004). Some of the sera reacted with autoantigens that are not usually associated with PBC, including CALR, GRIPAP1, SART1, and U1A (Bringmann et al. 1983; McCauliffe et al. 1990; Shichijo et al. 1998; Stinton et al. 2005). The remaining antigens, arranged in Table 1 using the Gene Ontology Consortium annotation system (Ashburner et al. 2000), include RNA-binding proteins, kinases and phosphatases, proteins involved in protein biosynthesis, and miscellaneous proteins. Thirteen additional antigens, not listed in Table 1, include unidentified proteins ($n = 5$) and uncharacterized, potential open reading frames ($n = 8$). Y-box protein 1 (YB-1) was chosen for further investigation because it

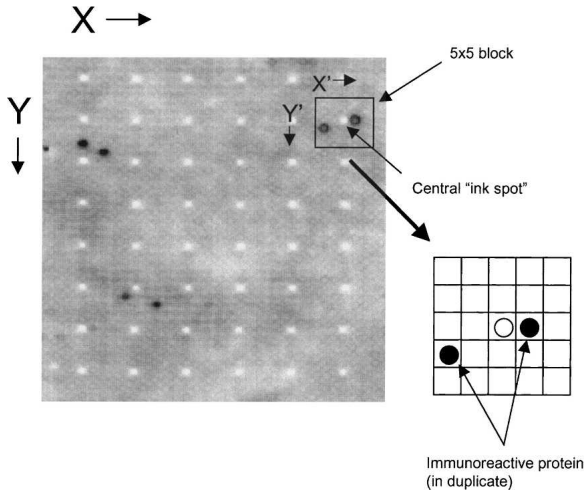

FIGURE 1. Antibodies in the serum of primary biliary cirrhosis patients react with proteins on a macroarray. The macroarray consists of 2304 blocks arranged in a 48-by-48 array. Each block consists of 24 squares surrounding a central ink spot. Twelve proteins are present, in duplicate, in each block. A section of the film produced by probing the macroarray with serum from a patient with PBC is shown. Detection of an immunoreactive protein requires the presence of two positive spots within a block, as indicated. The coordinates of an immunoreactive protein are determined by the X and Y axes of the blocks and the x′ and y′ axes of the positive dots within each block.

TABLE 1. Proteins identified on a macroarray using human sera containing anti-P-body autoantibodies

		Patient						
Accession #	Category/protein	Ge	004	017	038	061	080	121
	P-body components							
NM_018403	DCP1a	N	P	N	N	N	N	N
NM_014329	Ge-1/HEDLS/EDC4	P	P	P	N	N	N	P
NM_015578	RAP55	P	P	N	P	N	P	N
NM_003407	TTP/ZFP36	P	N	N	N	N	N	N
NM_004559	YB1	N	P	P	P	N	P	N
	PBC autoantigens							
NM_000108	E3 PDC/DLD	P	P	P	N	N	N	P
NM_170708	LMNA	N	P	P	N	P	P	N
NM_004559	VCP	N	P	P	N	P	P	P
	Other autoantigens							
NM_004343	CALR	N	N	N	N	N	N	P
NM_020137	GRIPAP1	P	P	P	P	P	P	P
NM_005146	SART1	P	P	N	P	P	P	P
NM_004596	U1A	P	N	P	P	P	P	P
	RNA-binding							
NM_007158	CSDE1	N	N	N	N	N	P	P
NM_015033	FNBP1	N	P	N	N	N	N	N
NM_004960	FUS	P	N	N	N	N	N	N
NM_031372	HNRPDL	N	P	N	N	P	N	P
NM_031266	HNRPAB	P	P	P	P	P	P	P
NM_004501	HNRPU	N	P	N	P	P	N	P
NM_005792	MPHOSPH6	P	P	P	P	N	P	P
NM_017948	NOL8	N	N	N	P	N	P	N
NM_002819	PTPB1	P	P	P	P	P	P	P
NM_006753	SURF6	P	P	P	P	P	P	N
	Protein biosynthesis							
NM_001961	EEF2	P	P	P	P	P	P	P
NM_015904	EIF5B	P	P	P	P	P	P	P
	Helicase							
NM_175066	DDX51	P	P	P	P	P	P	P
NM_018183	SBNO1	N	N	P	N	N	N	N
	Kinase/phosphatase							
NM_004418	DUSP2	N	P	P	P	P	N	P
NM_014330	PPP1R15A	N	N	N	P	N	N	N
NM_006244	PPP2R5B	N	P	N	N	N	N	N
NM_002719	PPP2R5C	N	P	P	P	P	P	N
NM_005026	PIK3CD	N	P	N	N	N	P	N
NM_002831	PTPN6	N	N	P	N	N	N	N
	Transcription							
NM_012138	AATF	P	P	P	N	P	P	P
NM_003655	CBX4	P	P	P	P	P	P	P
NM_022466	IKZF5	N	P	P	P	P	P	P
NM_002360	MAFK	P	P	P	P	P	P	P
NM_003926	MBD3	N	P	P	P	P	N	P
NM_001040443	PHF11	P	N	N	N	N	N	N
NM_002624	PFDN5	N	P	N	N	N	N	N
NM_021975	RELA	N	P	P	P	P	P	P
NM_013376	SERTAD1	P	P	P	N	N	P	P
NM_003086	SNAPC4	N	N	N	P	P	N	P
NM_024061	ZNF655	N	N	N	N	N	N	P

(*continued*)

TABLE 1. *Continued*

Accession #	Category/protein	Patient						
		Ge	004	017	038	061	080	121
	Miscellaneous							
NM_006796	AFG3L2	N	P	P	N	N	N	N
NM_004886	APBA3	P	P	P	P	P	P	N
NM_018690	APOB48R	P	P	P	P	P	P	P
NM_00333	ATXN7	N	P	P	N	N	N	N
NM_004281	BAG3	N	P	N	N	N	N	N
NM_001919	DCI	N	N	N	P	N	N	N
NM_004082	DCTN1	N	P	P	N	P	N	N
NM_024329	EFHD2	N	P	N	N	N	N	N
NM_002569	FURIN	P	N	N	P	N	N	N
NM_015710	GLTSCR2	P	P	N	P	P	N	N
NM_000852	GSTP1	N	P	N	N	N	N	N
NM_002117	HLA-C	P	P	P	P	P	P	P
NM_015190	HSP40	P	P	P	P	P	P	P
NM_021070	LTBP3	P	P	P	P	P	P	P
NM_002473	MYH9	N	P	P	P	P	P	P
NM_007346	OGFR	N	N	P	N	N	N	N
NM_194248	OTOF	N	N	P	N	N	N	N
NM_017884	PINX1	P	N	N	N	N	N	N
NM_017647	FTSJ3	P	P	P	P	N	P	P
NM_003612	SEMA7A	N	P	P	P	P	N	P
NM_005412	SHMT2	P	N	N	N	N	N	N
NM_014350	TNFAIP8	N	P	P	N	P	N	P
NM_000391	TPP1	N	N	P	N	N	P	N
NM_001008563	USP20	N	P	P	P	P	P	P

Abbreviations: P, immunoreactive; N, nonreactive.

contains RNA-binding domains and because it was previously shown to have a role in regulating mRNA stability (Evdokimova et al. 2001; Nekrasov et al. 2003).

Y-box protein 1 is a component of mRNA processing bodies

YB-1 is a 50-kDa protein that is the predominant component of translationally inactive mRNA-ribonucleoprotein particles (Minich et al. 1989; Evdokimova et al. 1995). YB-1 stabilizes mRNAs that have a 5′ cap but lack the eIF4e cap-binding protein (Evdokimova et al. 2001; Nekrasov et al. 2003). YB-1 may protect message from degradation until readdition of eIF4e and return of the mRNA to active translation in polysomes. Overexpression of YB-1 represses mRNA translation and increases mRNA stability. Depletion of YB-1 results in accelerated mRNA decay (Evdokimova et al. 2001).

YB-1 consists of an alanine- and proline-rich N-terminal domain (amino acids 1–55), followed by a cold shock domain (56–128) and a C-terminal region that contains four clusters of basic and acidic amino acids (129–324) (for review, see Kohno et al. 2003). The cold shock domain binds to both DNA and RNA. The C terminus of YB-1 also binds DNA and RNA and mediates protein–protein interactions. The functions of the YB-1 N terminus are unknown.

To investigate the cellular location of YB-1, a plasmid encoding green fluorescent protein (GFP) fused to the N terminus of YB-1 was transfected into Hep-2 cells. In cells expressing GFP–YB-1, antibodies directed against GFP localized to cytoplasmic dots and colocalized with antibodies directed against DCP1a (Fig. 2, panels a–c). To consider the possibility that GFP contributed to the localization of YB-1 to P-bodies, a plasmid encoding FLAG epitope fused to YB-1 was prepared and transfected into cells. FLAG–YB-1 was also detected in mRNA P-bodies (Fig. 2, panels d–f). To demonstrate that endogenous YB-1 also localizes to P-bodies, cells were stained with rabbit anti-YB-1 antiserum and human serum 121. As determined using the protein macroarray, this human serum reacted with Ge-1, but not YB-1. In addition to staining mRNA P-bodies, serum 121 also stained 5–20 dots in the nucleus of Hep-2 cells, reflecting the presence of autoantibodies directed against promyelocytic leukemia (PML) nuclear bodies (Fig. 2, panel h). Rabbit anti-YB-1 antiserum stained P-bodies but did not react with PML nuclear bodies (Fig. 2, panel g). Taken together, these results indicate that endogenous YB-1 is a component of P-bodies.

To identify the portion of YB-1 that directs the protein to P-bodies, expression vectors encoding GFP fused to various segments of YB-1 were transfected into Hep-2 cells. Results are summarized in Figure 3A, and representative

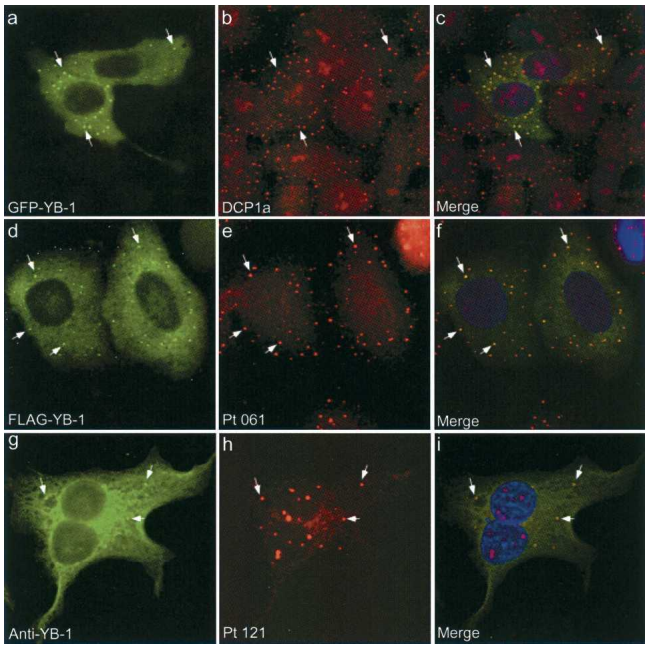

FIGURE 2. YB-1 localizes to mRNA processing bodies. Indirect immunofluorescence was used to show that YB-1 localizes to P-bodies. GFP–YB-1 (green, panel a) localized to discrete, dot-like structures in the cytoplasm of transfected Hep-2 cells and colocalized with P-bodies recognized by rabbit anti-DCP1a antiserum (red, panel b). FLAG–YB-1 also localized to cytoplasmic dots (green, panel d) and colocalized with P-bodies identified using serum from patient 061 (red, panel e). To determine whether endogenous YB-1 also localized to P-bodies, rabbit anti-YB-1 antiserum (green, panel g) and human serum 121 (red, panel h) were used to stain Hep-2 cells. Serum 121 reacted with both P-bodies and PML nuclear bodies. In contrast, rabbit anti-YB-1 antiserum detected YB-1 within P-bodies and diffusely throughout the cytoplasm, but did not stain PML-nuclear bodies. White arrows indicate the location of representative P-bodies in transfected cells. DAPI staining (blue) indicates the location of nuclei. Overlap of red, green, and blue staining is shown in panels c, f, and i.

photomicrographs are shown in Figure 3B. Successful production of GFP–YB-1 fusion proteins was confirmed by immunoblot using rabbit anti-GFP antiserum (data not shown). A GFP fusion protein containing YB-1 amino acids 1–281 localized to P-bodies, showing that the C-terminal 43 amino acids were not required for P-body localization (Fig. 3B, panels a–c). N-terminal (1–205) and C-terminal (128–324) segments of YB-1 were detected in nuclei and nucleoli and did not localize to P-bodies (Fig. 3B, panels d–i). Jurkott and colleagues and Bader and Vogt observed similar staining patterns using N- and C- terminal fragments of YB-1 (Jurchott et al. 2003; Bader and Vogt 2005). A portion of YB-1 (55–324) that lacked the N-terminal domain was present throughout transfected cells and was not concentrated in P-bodies (data not shown). These results indicate that the alanine-proline-rich N terminus, the cold shock domain, and amino acids 205–281 of the C terminus are required for P-body localization.

YB-1 localizes to TIA-containing stress granules

To investigate the cellular location of YB-1 in response to environmental stress, GFP–YB-1 was transfected into Hep-2 cells and the cells were exposed to arsenite for 1 h. Under these conditions, YB-1 colocalized with TIA in stress granules (Fig. 4A, panels a–c). Stress granules appeared larger and more numerous in YB-1 transfected cells, suggesting that YB-1 may contribute to stress granule formation and/or increase the stability of these structures. However, in control Hep-2 cells, expression of YB-1 did not alter the nuclear location of TIA, suggesting that YB-1 did not induce stress granule formation in the absence of oxidative stress (Fig. 4A, panels d–f). Note that endogenous YB-1, identified using rabbit anti-YB-1 antiserum, also localized to stress granules in response to oxidative stress (Fig. 4A, panels g–i).

To identify the portion of YB-1 that is required for localization to stress granules, Hep-2 cells were transfected with plasmids encoding GFP fused to DNA encoding portions of the protein. Although a fusion protein (GFP–YB-1 [55–324]) that lacked the YB-1 N-terminal domain was unable to localize to P-bodies, this fusion protein was capable of localizing to stress granules (Fig. 4B, panels a–c). In contrast, a fusion protein (GFP–YB-1 [128–324]) that lacked both the N-terminal domain and the cold shock domain did not associate with stress granules (Fig. 4B, panels d–f). A fusion protein (GFP–YB-1 [1–205]) that contained the N-terminal and cold shock domains localized to stress granules, but a fusion protein (GFP–YB-1 [1–86]) that lacked the C-terminal 42 amino acids of the cold shock domain did not localize to these structures (data not shown). A small segment of YB-1 (amino acids 44–128), containing the cold shock domain, was sufficient to direct GFP to stress granules (Fig. 4B, panels g–i). These results indicate that neither the N-terminal nor the C-terminal domain of YB-1 was required for stress granule localization.

In a previous study, we showed that P-body component RAP55, like YB-1, is capable of translocating from P-bodies to stress granules (Yang et al. 2006). To examine the cellular location of YB-1 compared to that of RAP55 during arsenite-induced stress, cells were transfected with plasmids encoding FLAG–YB-1 and GFP–RAP55 and were exposed to arsenite. Both YB-1 and RAP55 were detected in stress granules (Fig. 4C, panels a–c). To determine the cellular location of YB-1 during recovery from stress, YB-1- and RAP55-transfected cells were exposed to arsenite for 1 h, rinsed with PBS, and then permitted to recover in culture medium for 1, 3, 5, or 12 h. One hour after removal of arsenite, RAP55 was detected both within stress granules and in adjacent P-bodies (Fig. 4C, panel d). In contrast, YB-1 persisted in stress granules (Fig. 4C, panel e). After 3 h of recovery from stress, RAP55 was detected within P-bodies but was no longer present in stress granules. YB-1 remained in stress granules or was distributed diffusely throughout the cytoplasm, but was not detected

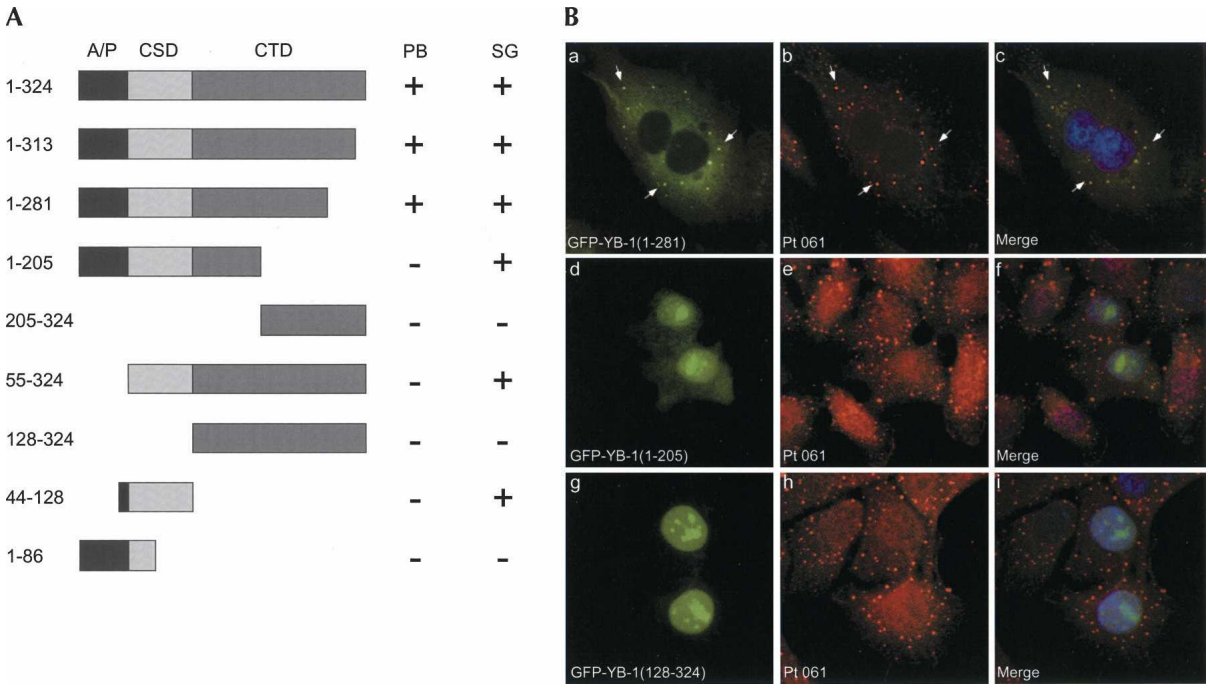

FIGURE 3. Structure of YB-1 and identification of the portions of YB-1 that mediate localization to P-bodies. (*A*) The predicted amino acid sequence of YB-1 contains an N-terminal alanine-proline rich region (A/P) and a cold shock domain (CSD). The C-terminal domain (CTD) contains alternating basic and acidic segments. The portions of YB-1 that were capable of localizing green fluorescent protein (GFP) to P-bodies (PB) or stress granules (SG) are indicated by "+." Representative indirect immunofluorescence results are shown in *B*. (*B*) Indirect immunofluorescence was used to identify the portion of YB-1 that was necessary for localization to P-bodies. A segment of YB-1 containing amino acids 1–281 (green, panel a) colocalized with P-bodies that were identified using serum from patient 061 (red, panel b). White arrows indicate representative P-bodies. N-terminal (1–205) and C-terminal (128–324) portions of YB-1 localized to nuclei and nucleoli (green, panels d and g) but did not localize to P-bodies (red, panels e and h). DAPI staining (blue) indicates the location of nuclei. Merge of red, green, and blue staining is shown in panels c, f, and i.

in P-bodies (data not shown). At 5 and 12 h after removal of arsenite, both RAP55 and YB-1 were detected in P-bodies (data not shown). The differing cellular location of RAP55 and YB-1 during the early stages of recovery from stress suggests that these proteins may have different roles in the "sorting" of mRNAs that occurs in stress granules. RAP55 may escort some mRNAs from stress granules to P-bodies. In contrast, YB-1 may transfer other mRNAs from stress granules back to polyribosomes.

Conclusions

In this study, we used protein macroarray technology and human autoantibodies to identify four known, and 63 potential, components of mRNA P-bodies. The possible relationship between the majority of these antigens and P-bodies remains to be determined. We showed that one of these proteins, YB-1, does localize to P-bodies. The alanine-proline-rich N terminus, the cold shock domain, and the N-terminal half of the C-terminal domain were required to target YB-1 to P-bodies. A much smaller portion of the protein, which contained the cold shock domain, was necessary and sufficient to target the protein to stress granules. YB-1 is similar to RAP55 in that it translocates to stress granules in response to oxidative stress. In contrast to RAP55, YB-1 does not return to P-bodies during the immediate recovery period.

YB-1 protects mRNA from degradation and is particularly effective at mRNA stabilization when it binds, in the absence of eIF4e, to the 5′ cap. We speculate that the role of YB-1 in P-bodies may be to protect mRNA from degradation, possibly allowing readdition of eIF4e and return of the mRNA to polysomes. Consistent with this model, YB-1 may be excluded from P-bodies during recovery from stress because, during this critical period, P-bodies are actively involved in the destruction of damaged mRNA.

This is the first study to use protein macroarrays and human autoantibodies to define the composition of a cellular structure. We anticipate that the combination of macroarrays and autoantibodies will prove to be an efficient approach to identifying additional components of P-bodies and that "autoantigenomics" may be applied to the study of other cellular domains.

MATERIALS AND METHODS

Plasmids and antisera

A plasmid encoding YB-1 was obtained from the German Resource Center for Genome Research (RZPD, Berlin, Germany;

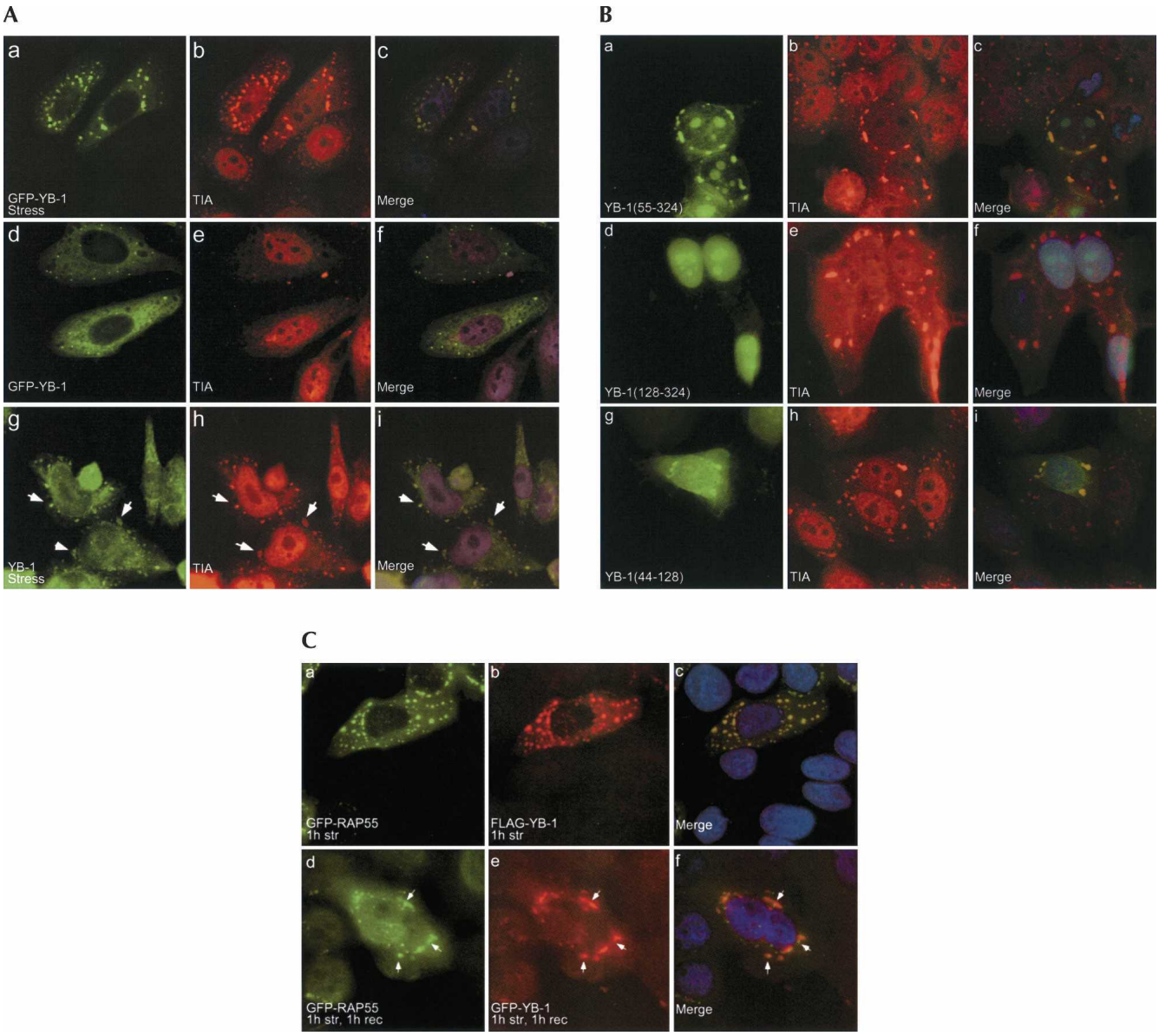

FIGURE 4. YB-1 localizes to stress granules. (*A*) A plasmid encoding GFP–YB-1 was transfected into Hep-2 cells and cells were exposed to arsenite for 1 h. In transfected cells, GFP–YB-1 (green, panel a) colocalized with TIA (red, panel b) in stress granules. In control cells, expression of GFP–YB-1 (green, panel d) did not induce stress granule formation (red, panel e). Endogenous YB-1 (green, panel g) also localized to stress granules (red, panel h) in response to oxidative stress. White arrows in panels g–i indicate the location of representative stress granules. DAPI staining (blue) indicates the location of nuclei. Merge of red, green, and blue is shown in panels c, f, and i. (*B*) A fusion protein (GFP–YB-1 [55–324]) that lacked the N-terminal domain of YB-1 (green, panel a) localized to stress granules (red, panel b). A fusion protein (GFP–YB-1 [128–324]) that lacked both the N-terminal domain and the cold shock domain (green, panel d) did not localize to stress granules (red, panel e). A fragment of YB-1 that contained the cold shock domain (44–128) (green, panel g) was sufficient to direct GFP to stress granules (red, panel h). DAPI staining (blue) indicates the location of nuclei. Merge of red, green, and blue staining is shown in panels c, f, and i. (*C*) To examine the cellular location of YB-1 compared to that of RAP55 during arsenite-induced stress, cells were cotransfected with GFP–RAP55 (green, panel a) and FLAG–YB-1 (red, panel b) and exposed to arsenite. Both YB-1 and RAP55 were detected in stress granules. In cells exposed to arsenite and then permitted to recover for 1 h, RAP55 was detected within stress granules and within adjacent P-bodies (green, panel d). In contrast, YB-1 (red, panel e) persisted in stress granules. White arrows in panels d–f indicate the presence of P-bodies (panels d and f) or absence of P-bodies (panel e) adjacent to stress granules. DAPI staining (blue) indicates the location of nuclei. Merge of red, green, and blue staining is shown in panels c and f.

catalogue number: RZPDp9016E0814). The cDNA was ligated into pEGFP (Clontech) and pCMV-FLAG (Invitrogen) to produce green fluorescence protein (GFP)–YB-1 and FLAG–YB-1 fusion proteins, respectively. Plasmids encoding GFP fused to amino acids 1–313, 1–281, 1–205, and 1–86 were prepared using YB-1 cDNA restriction sites *EcoR*I, *Kpn*I, *Sal*I, and *Bsrg*I, respectively. A plasmid encoding YB-1 amino acids 205–324 was prepared by ligating YB-1 cDNA *Sal*I fragment into pEGFP. PCR was used to

prepare DNA encoding YB-1 amino acids 55–324, 128–324, and 44–128. The nucleotide sequence of each PCR product was confirmed by DNA sequencing. A plasmid encoding GFP fused to RAP55 was previously described (Yang et al. 2006).

Sera from 34 patients with anti-P-body antibodies were identified during the course of studies to determine the clinical significance of autoantibodies in patients with PBC (Yang et al. 2004). Ge serum and patient sera 080 and 017 were previously used to identify and characterize P-body components Ge-1 and RAP55 (Yu et al. 2005; Yang et al. 2006). The remaining four sera (004, 038, 061, and 121) produced a typical P-body staining pattern. Two of the seven patient sera (Ge and 121) reacted with PML nuclear bodies, as well as P-bodies.

Rabbit anti-DCP1a and anti-YB-1 antisera were provided by J. Lykke-Andersen (University of Colorado, Boulder, CO) and P.E. Newburger (University of Massachusetts, Worcester, MA), respectively. Mouse and rabbit anti-GFP antisera were obtained from Invitrogen. Mouse anti-FLAG antiserum was purchased from Sigma-Aldrich. Goat anti-TIA antiserum was obtained from Santa Cruz Biotechnology. FITC- or rhodamine-conjugated, species-specific, donkey anti-human, anti-goat, anti-rabbit, and anti-mouse IgG antisera were obtained from Jackson ImmunoResearch Laboratories.

Cell culture and immunohistochemistry

Hep-2 cells were obtained from ATCC and maintained in DMEM supplemented with 10% Nuserum (BD Bioscience), L-glutamine (2 mM), penicillin (200 U/mL), and streptomycin (200 μg/mL). For immunofluorescent staining, Hep-2 cells were grown in tissue culture chambers (Nunc Inc.), fixed with 2% paraformaldehyde in PBS, and permeabilized with 100% methanol. Cells were stained with primary and secondary antisera as previously described (Bloch et al. 2005).

Screening the high-density protein macroarray

High-density protein macroarrays derived from human T-cells treated with PHA were obtained from RZPD. Membranes were initially rinsed in 100% ethanol at room temperature, incubated in blocking solution (3% nonfat, dry milk powder in TBST [TBS, 0.1% v/v Tween 20]) for 2 h and washed twice in TBST. Membranes were then incubated overnight at 4°C with human serum diluted 1:3000 in blocking solution. Bound antibodies were detected using horse-radish peroxidase-conjugated rabbit anti-human IgG antiserum (Sigma-Aldrich) and chemiluminence.

Mammalian cell transfection

To identify the portion of YB-1 that mediates localization to P-bodies, plasmids encoding GFP fused to segments of YB-1 were transfected into Hep-2 cells using the Effectene transfection system (Qiagen) as directed by the manufacturer. Cells were fixed and stained 24 h after transfection. To identify the portion of YB-1 that mediates stress granule localization, transfected cells were exposed to sodium arsenite (0.5 mM) for 1 h at 37°C. To determine the cellular location of YB-1 during recovery from stress, arsenite-containing medium was removed and cells were washed with PBS and incubated in culture medium. Cells were permitted to recover for 1, 3, 5, and 12 h.

ACKNOWLEDGMENTS

We thank J. Lykke-Andersen and P.E. Newburger for providing rabbit anti-DCP1a and anti-YB-1 antisera, respectively. S. Hennig (RZPD, Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany) assisted in the identification of proteins on the macroarrays. D.B.B. was supported by grants from the Arthritis Foundation, the Milton Fund, and the National Institutes of Health (DK051179).

Received November 29, 2006; accepted February 2, 2007.

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