Arch. Biol. Sci., Belgrade, 61 (4), 683-691, 2009 DOI:10.2298/ABS0904683K

CHARACTERIZATION OF ANTIBODIES DIRECTED AGAINST THE ANKRD2 HUMAN MUSCLE PROTEIN

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Abstract — In order to study the function of the Ankrd2 protein, for which commercial antibodies are not available, we report the production and analysis of polyclonal antibodies to full-length Ankrd2 and its C-terminal and N-terminal regions, as well as a monoclonal antibody to the C-terminus of the protein. Epitope mapping making use of recombinant deletion mutants showed that an epitope located in region 323-333 aa of Ankrd2 is detected by the monoclonal antibody. The high specificity of all four anti-Ankrd2 antibodies for recombinant and endogenous Ankrd2 protein is also demonstrated.

Key words: Antibody, epitope, ankyrin repeats, muscle protein

UDC 572.2:575

INTRODUCTION

Three closely related proteins – CARP (cardiac ankyrin repeat protein; Ankrd1 or MARP1), Ankrd2 (ankyrin repeat domain 2; Arpp or MARP2), and DARP (diabetes-related ankyrin repeat protein; MARP3) – have been allocated to the muscle ankyrin repeat protein (MARP) family (Miller et al., 2003). Muscle ankyrin repeat proteins are characterized by modular structure, muscle-specific expression, and dual cellular localization (nuclear and cytoplasmic). They can play important structural and signalling roles and link the elastic I-band region as a passive stretch sensor to the control of transcription.

Kemp et al. (2000) identified Ankrd2 as a novel transcript expressed in fast tibialis anterior muscles after 7 days of passive stretch immobilization *in vivo*. In addition to this, Ankrd2 was also discovered through systematic sequencing of human skeletal muscle 3′-expressed sequence tags (ESTs) (Lanfranchi et al., 1996), showing 43% identity at the amino acid level to CARP, a nuclear protein expressed in heart (Zou et al., 1997), endothelial cells (Chu et al., 1995) and (to a lesser extent) in skeletal muscle (Baumeister et al., 1997).

The human Ankrd2 gene encodes a 37-kDa protein that has a nuclear localization signal (NLS), two PEST protein-destabilization motifs, and four ankyrin-repeat domains (Kemp et al., 2000; Moriyama et al., 2001; Pallavicini et al., 2001). Ankyrin repeats are ubiquitous motifs involved in macromolecular interactions, whereas PEST sequences (rich in Pro, Glu, Asp, Ser, and Thr) serve as signals that target proteins for rapid destruction. Although Ankrd2 is predominantly expressed in skeletal muscle, in the I band of the sarcomere (Tsukamoto et al., 2002), it is also found in adult heart (Pallavicini et al., 2001; Ishiguro et al., 2002). It becomes strongly upregulated in myocytes upon stretch (Kemp et al., 2000), with denervation of skeletal muscle (Tsukamoto et al., 2002), and during eccentric exercise (Barash et al., 2004), suggesting its role in muscle cell response to acute stress.

It has been shown that Ankrd2 can interact with the Z-disk proteins titin (Miller et al., 2003) and telethonin (Kojic et al., 2004), as well as with three transcription factors: YB1, PML, and p53 (Kojic et al., 2004). The observation that Ankrd2 can interact with proteins located both in the nucleus and in the Z-disk supports the hypothesis of its potential role

as a molecular messenger shuttling between the nucleus and the cytoplasm. Not only does Ankrd2 bind p53, both *in vitro* and *in vivo*, it also enhances upregulation of the $p21^{WAFI/CIPI}$ promoter by p53 (Kojic et al., 2004). These findings suggest that Ankrd2 may be involved in sensing stress signals and linking these to muscle gene regulation.

In this paper, we describe a preparation of four anti-Ankrd2 antibodies (three polyclonal and one monoclonal) and precise mapping of their epitopes in order to define their specificity for the Ankrd2 protein. In view of the dual localization of Ankrd2 in muscle cells and the presence of a common motif such as ankyrin repeats, it is important to determine that anti-Ankrd2 antibodies specifically recognize the Ankrd2 protein and no other protein containing ankyrin repeats in muscle and heart tissue.

MATERIALS AND METHODS

Constructs

Full-length (FL-Ankrd2, 13-999 bp) and C1-Ankrd2 (721-999 bp) cDNAs were amplified by PCR, while N1-Ankrd2 (13-519 bp) cDNA was generated by restriction digestion (KpnI and HindIII) of full-length Ankrd2 cDNA. All three cDNAs were inserted into the pQE30 expression vector (Qiagen) in frame with the 6-Histidine tag.

Further, FL-Ankrd2 (13-999 bp), N-Ankrd2 (13-360 bp), NA-Ankrd2 (13-852bp), C-Ankrd2 (839-999 bp), AC-Ankrd2 (361-999 bp), DC-Ankrd2 (13-969 bp), UC-Ankrd2 (13-948 bp), and SC-Ankrd2 (949-999 bp) cDNAs were amplified by PCR and cloned into the pGEX-6P-3 vector (GE Healthcare) in frame with the GST tag.

Expression and purification of His-tagged recombinant proteins

His-tagged recombinant proteins were produced using the QIAexpress system (QIAGEN). The Ankrd2 constructs in pQE30 were transformed in *E. coli* strain M15 carrying pREP4. Bacteria were grown in LB containing 100 μg/ml ampicillin and 30 μg/ml kanamycin. Expression from the pQE30 vector was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation and the pellet was resuspended in lysis buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8). After sonication, lyzates were cleared by centrifugation at 12000g at +4°C for 10 min and mixed with Ni-NTA (QIAGEN) resins. Following 1-h incubation, resins were washed with washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) and the recombinant protein was eluted with elution buffer containing EDTA (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, 100 mM EDTA, pH 6.3). Protein levels and purity were checked by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Polyclonal antibody production

His-tagged recombinant proteins, purified under denaturing conditions, were used for immunization of BALB/c mice. Prior to the first injection, blood was taken from the carotid artery; pre-immune sera were prepared and tested by Western blot in order to check for any reactivity against muscle proteins.

Mice were immunized with 40 μg of purified proteins [diluted in PBS and mixed with Freund's incomplete adjuvant (SIGMA)] by intra-peritoneal injection. Every three weeks mice were injected with proteins. After three injections, a blood sample was taken; thereafter boosts were given every three weeks and samples taken once every three weeks. Sera were prepared by allowing the blood to coagulate (37°C for 30 min) and then centrifuging at 12000g at 4°C for 30 min. To avoid the growth of bacteria and fungi, sodium azide was added to the sera to a final concentration of 0.02%. Samples were aliquoted and stored at -20°C. All collected sera were tested by Western blot analysis for immunoreactivity against human striated muscle proteins.

Monoclonal antibody production

A modified version of the protocol of Harlow and Lane (Harlow and Lane, 1998) was used for production of monoclonal antibodies. Here BALB/c female mice were immunized as already described for the production of polyclonal antibodies. When a good immune response was obtained, the animals were sacrificed. The spleen was taken and the cells disso-

ciated and then fused with NP1 myeloma cells in the presence of polyethylene glycol. After fusion, hybridoma cells were selected using RPMI medium supplemented with sodium hypoxanthine aminopterin and thymidine (HAT, SIGMA). Clones were tested by enzyme-linked-immunosorbent assay (ELISA) against the Ankrd2 protein. In order to obtain monoclonal cell lines, positive clones were subcloned, tested by ELISA, and then grown in DMEM supplemented with HAT. These monoclonal cell lines were tested by Western blot analysis for immunoreactivity against striated muscle proteins.

Expression and purification of GST-tagged recombinant proteins

Production of GST-tagged recombinant proteins and their purification were accomplished using the Glutathione-S-transferase Gene Fusion System (GE Healthcare). The Ankrd2 constructs in pGEX-6P-3 expression vector were transformed in *E. coli* strain BL21(DE3)-pLys S (Promega) and grown in LB with ampicillin (100 μg/ml). The GST-recombinant protein expression was induced by adding IPTG to a final concentration of 0.5-1 mM and the bacteria were grown for an additional 3 h at room temperature. Cells were pelleted by centrifugation, resuspended in lysis buffer [50 mM Tris-HCl, pH 8, 5% glycerol, 1% Triton X-100, 2 mM DTT, and a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche)], and sonicated. Debris from the cell lyzate was removed by centrifugation and the supernatant was incubated with Glutathione Sepharose-4CLB beads (GE Healthcare) for 1 h at 4°C. After removal of non-specifically bound proteins by washing resins with PBS [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and a cocktail of protease inhibitors (Complete Mini, EDTA-free, Roche)], the GST-recombinant proteins were eluted from the beads with 20 mM glutathione dissolved in 50 mM Tris-HCl, pH 8. Quality and quantity of the purified proteins were evaluated by SDS-PAGE followed by Coomassie Brilliant Blue staining.

Western blot analysis

Recombinant GST-tagged proteins or human heart (Human Heart Protein Medlay, Clontech) and skeletal muscle proteins (Human Skeletal Muscle Protein Medley, Clontech) were separated by SDS-PAGE and blotted onto PVDF membrane (Immobilon P, Millipore). The membrane was incubated in blocking solution [10% non-fat dried milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0,1% (v/v) Tween 20, pH 7.5)] at 4°C overnight, followed by 1-h incubation with each of the primary or secondary antibodies diluted in 5% non-fat dried milk in PBS-T. Detection of Ankrd2 was with mouse monoclonal antibody and mouse polyclonal antibodies to full length, the N-terminus, and the C-terminus of Ankrd2. All antibodies were diluted 400 times. Both alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated secondary antibodies were used for protein visualization, depending on the detection method employed: the NBT/BCIP colorimetric detection system (Promega, Milan, Italy) or the ECL detection reagent (Millipore, Milan, Italy).

RESULTS

Antigen and antibody preparation

In order to produce antibodies to the Ankrd2 protein, the full-length (5-333 aa) protein and two fragments of Ankrd2, N1-Ankrd2 (5-173 aa) and C1-Ankrd2 (241-333 aa), were used as antigens. Polyclonal antibodies were raised against the whole protein (anti-FL-Ankrd2), as well as the N- (anti-N-Ankrd2) and C-terminal (anti-C-Ankrd2) regions of Ankrd2. Monoclonal antibody was prepared against the C-terminus of Ankrd2.

The antigens were expressed in *E. coli* as His-tagged recombinant proteins and used for immunization of mice. Spleen cells isolated from mice immunized with His-tagged C-terminal Ankrd2 were fused with NP1 myeloma cells and selected for hybridoma cells secreting Ankrd2 monoclonal antibody. Epitope mapping was performed for clone 2F10.

Mapping antigenic epitope for monoclonal anti-Ankrd2 antibody

Since the C-terminal region of Ankrd2 (241-333 aa) was used for the production of monoclonal antibod-

ies, we wanted to check for a potential epitope. We searched for regions that might be able to adopt a conformation with exposed secondary structure, thereby representing potential antigen epitopes. The sequence of the Ankrd2 region from 241-333 aa was analyzed using ProtScale software (www.expasy.ch) (Gasteiger, 2005), which allows the computation and representation of the profile produced by any amino acid scale on a selected protein. The hydrophobicity scale was used here, and results of the prediction are presented in Fig. 1. Four regions (247-255 aa, 305-314 aa, 317-325 aa, and 320-328 aa) with the lowest scores are designated, suggesting the most hydrophilic regions that could represent potential epitopes. The same regions were also predicted to be the most hydrophilic when the Ankrd2 sequence from amino acid 241-333 was analyzed by ProtScale choosing the hydrophilicity scale (data not shown). In order to precisely map the antigenic epitope for monoclonal anti-Ankrd2 antibody several constructs were made with the aim of defining the minimal region of the Ankrd2 protein recognized by the anti-Ankrd2 monoclonal antibody. The following regions were selected: UC-Ankrd2 (5-316 aa), containing the first and second potential epitopes; DC-Ankrd2 (5-323 aa), containing the first, second, and third potential epitopes; SC-Ankrd2 (317-333 aa), containing third and fourth potential epitopes positioned in close proximity (Fig. 1); C-Ankrd2 (279-333 aa), containing second, third and fourth potential epitopes; and AC-Ankrd2 (121-333 aa), containing all four potential epitopes. All Ankrd2 fragments used to characterize the anti-Ankrd2 antibodies are shown schematically in Fig. 2. The full-length protein and deletion mutants of the Ankrd2 protein were expressed in *E. coli* as GST-tagged recombinant proteins and purified under native conditions. Equal quantities of purified proteins were separated by SDS-PAGE, and the proteins were visualized by staining with Coomassie Brilliant Blue (Fig. 3, panel A).

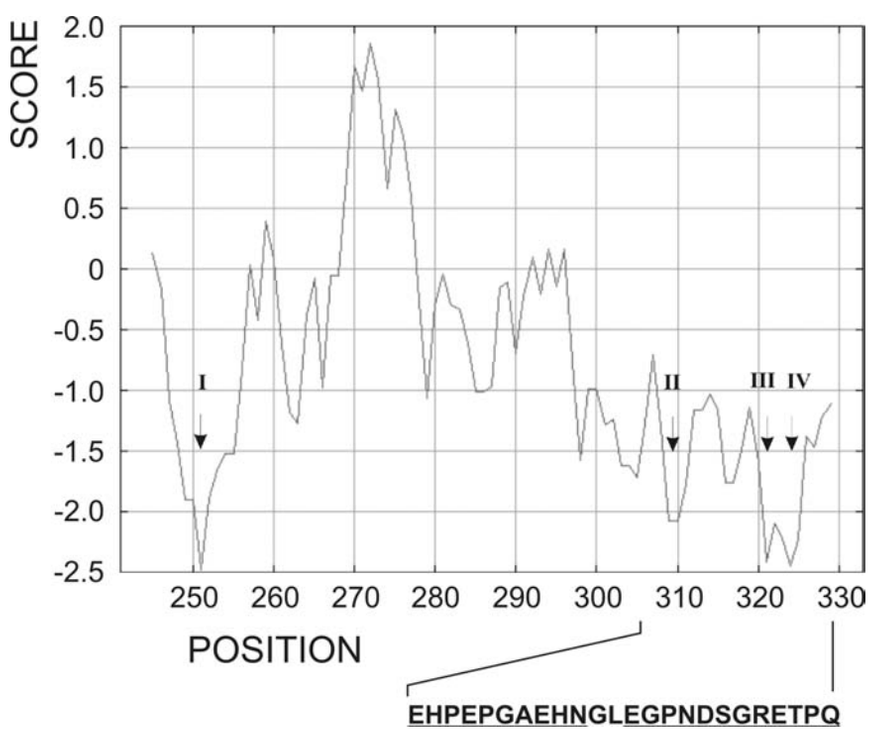

Fig. 1. ProtScale hydrophobicity graph for the Ankrd2 C-terminal region from amino acids 241 to 333. Arrows designate the peaks with minimal scores. The sequence of the region containing the second, third, and fourth potential epitope (310-333 aa) is given under the position axis. Sequences of the potential second, third, and fourth epitopes are underlined (the third and fourth regions are merged). Length of the interval (window size) used for profile computation was 9.

For Western blot analysis, proteins (1/50 of that used in panel A) were separated by SDS-PAGE, blotted, and probed with the monoclonal anti-Ankrd2 antibody, clone 2F10 (Fig. 3, panel C), as well as the anti-FL-Ankrd2 antibody (Fig. 3, panel B) as a positive control and the pre-immune mouse serum (Fig. 3, panel E) as a negative control.

The monoclonal antibody raised against the C-terminus of Ankrd2 interacted only with recombinant proteins containing the last 10 aa of the C-terminus, viz., C (279-333 aa), AC (120-333 aa), and SC (317-333 aa), but not with the Ankrd2 fragments UC (5-316 aa) and DC (5-323 aa) (Fig. 3, panel C). As expected, the antibody to the full-length protein could detect the proteins expressed by the all of the GST-Ankrd2 constructs, but not GST alone (Fig. 3, panel B). We therefore deduced that the monoclonal antibody (clone 2F10) epitope maps the region between amino acids 323 to 333, at the very end of the Ankrd2 protein.

Determination of specificity of the polyclonal anti-N-terminal and anti-C-terminal Ankrd2 antibodies

Antigens used for production of anti-N-terminal (5-173 aa) and anti-C-terminal (241-333 aa) Ankrd2 antibodies contained some of the ankyrin repeats (120-278 aa). To exclude the possibility that polyclonal anti-N-terminal antibody recognizes ankyrin repeats, we checked it against the same set of deletant mutants used to map the epitope of the monoclonal antibody, with the addition of an Ankrd2 fragment containing the N-terminal region of Ankrd2 (N, 5-120 aa, Fig. 2). To test the polyclonal antibody raised against the C terminus of Ankrd2, we used an Ankrd2 fragment containing the N-terminus and ankyrin repeats (NA, 5-278 aa, Fig. 2), as well as the C-terminus (C, 279-333 aa). Purified GST recombinant proteins were subjected to SDS-PAGE, blotted, and the blots probed with polyclonal anti-N-Ankrd2 (Fig. 3, panel D) and anti-C-Ankrd2 antibodies (Fig. 4).

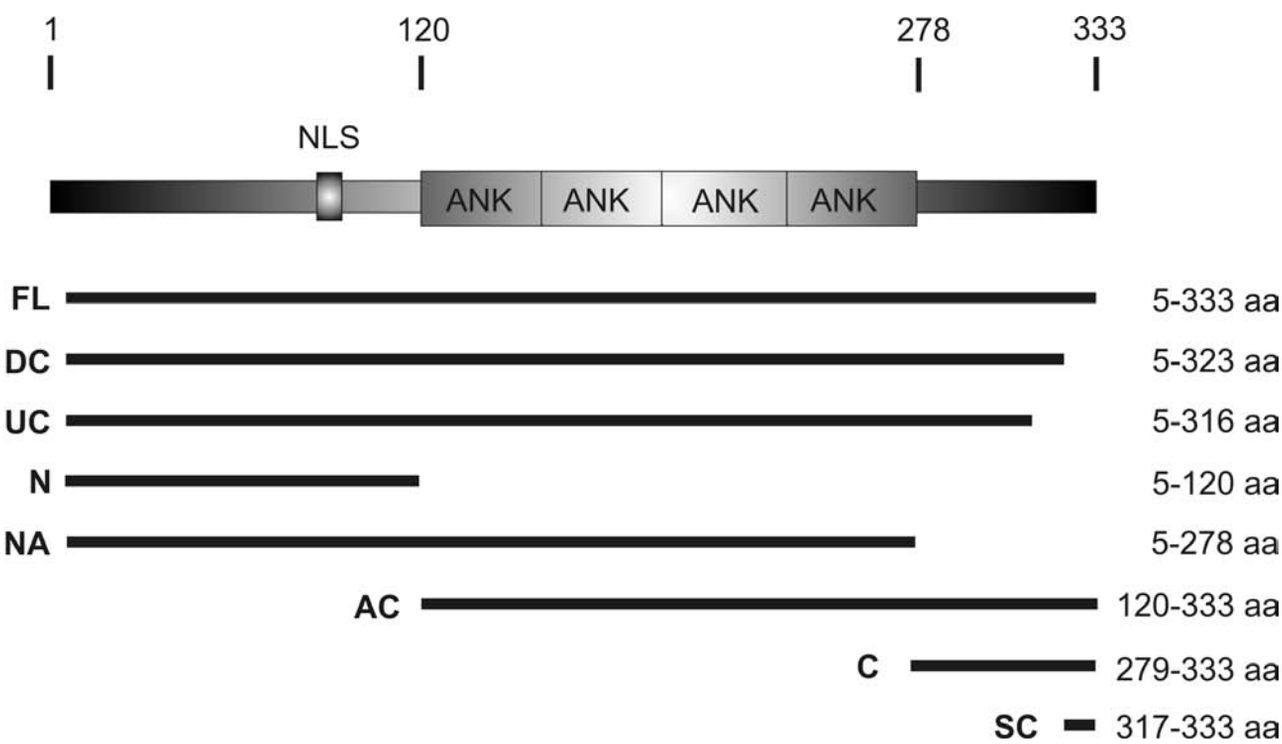

Fig. 2. Schematic representation of human Ankrd2 and its deletant mutants used to map antigenic epitopes. NLS, nuclear localization signal; ANK, ankyrin repeat domains.

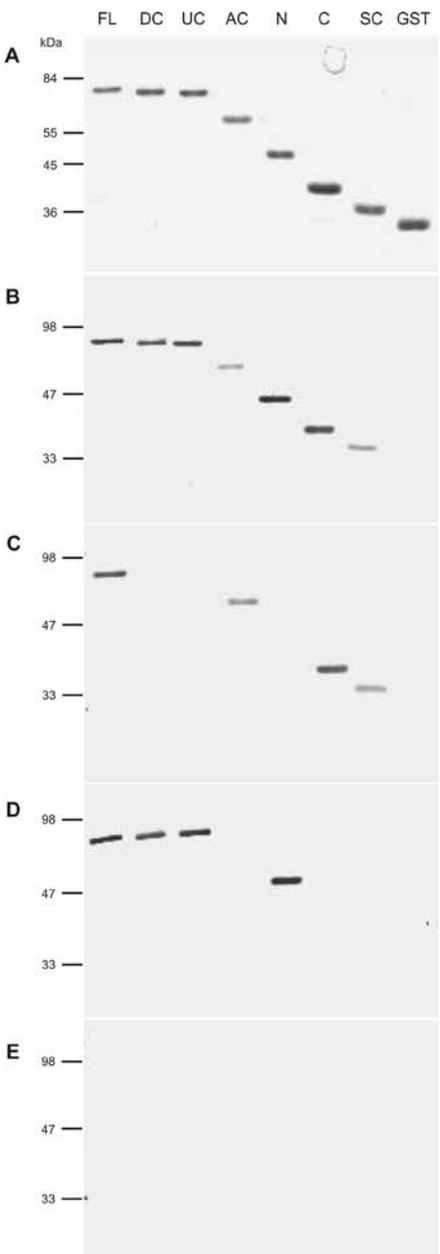

Fig. 3. Mapping of anti-Ankrd2 epitopes by Western blot analysis. Here GST-Ankrd2 (FL, aa 5-333) and its fragments tagged with GST (DC, aa 5-323; UC, aa 5-316; AC, aa 120-333; N, aa 5-120; C, aa 279-333; and SC, aa 317-333) were expressed in *E. coli* and purified under native conditions. The GST protein was used as a negative control. Equal amounts of purified proteins were separated by SDS-PAGE and the proteins were visualized by staining with Coomassie Brilliant Blue (panel A). For Western blot analysis, 1/50 of that used for visualization was separated by SDS-PAGE, blotted, and probed with anti-FL (panel B), monoclonal anti-Ankrd2, clone 2F10 (panel C), and anti-N-Ankrd2 (panel D) antibodies, as well as pre-immune mouse serum (panel E) as a negative control.

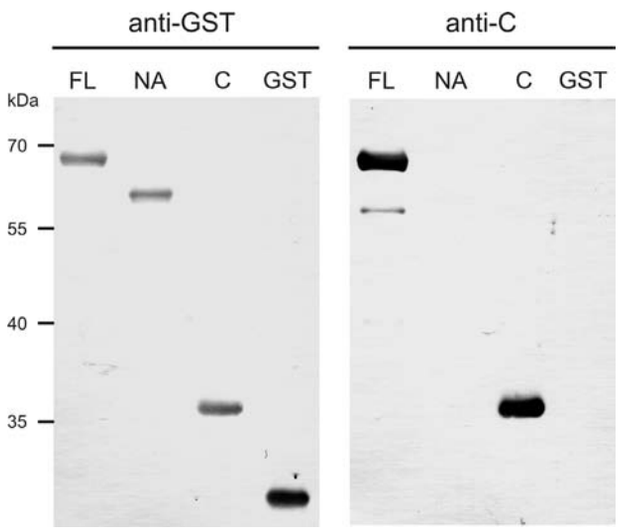

Fig. 4. Specificity of polyclonal anti-C-Ankrd2 antibody for the C-terminus of Ankrd2. Western blot analysis of GST-tagged Ankrd2 (FL, aa 5-333), the N-terminus with ankyrin repeats (NA, aa 5-278), and the C-terminus (C, aa 279-333). Purified recombinant proteins were separated by SDS PAGE, blotted, and probed with anti-GST and anti-C-Ankrd2 antibodies. The GST protein was used as a negative control.

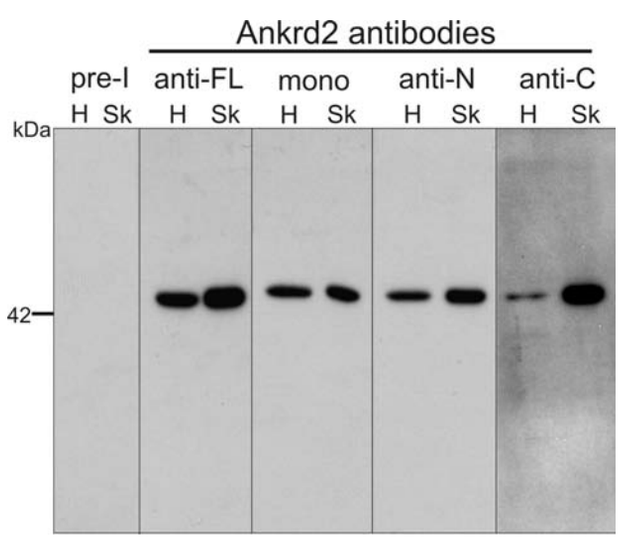

Fig. 5. Western blot analysis showing the specificity of anti-Ankrd2 antibodies for endogenous Ankrd2 protein. Here 20 μg of human heart (H) and 5 μg of skeletal muscle (Sk) proteins were separated by SDS-PAGE, blotted, and probed with pre-immune sera (pre-I), anti-FL-Ankrd2 (anti-FL), monoclonal anti-Ankrd2 (mono), anti-N-Ankrd2 (anti-N), and anti-C-Ankrd2 (anti-C) antibodies as indicated.

The C-terminal region of Ankrd2 with ankyrin repeats (AC) was not detected by the N-terminal polyclonal antibody (Fig. 3, panel D), suggesting that this antibody is specific for the N-terminal region of Ankrd2 and does not cross react with the ankyrin repeats.

Polyclonal antibody raised against the C-terminus of Ankrd2 reacted with the C-terminus of the Ankrd2 protein, but not with the N-terminus and ankyrin repeats (Fig. 4), thus demonstrating that this antibody is also specific for the Ankrd2 protein.

Anti-Ankrd2 antibodies recognize endogenous Ankrd2 in human heart and skeletal muscle

The specificity of all anti-Ankrd2 antibodies was tested by Western blot analysis of human heart and skeletal muscle proteins. The proteins were separated by SDS-PAGE and blotted. Endogenous Ankrd2 was detected by all four anti-Ankrd2 antibodies. Figure 5 shows a single 43-kDa band corresponding to endogenous Ankrd2, detected in both human heart and skeletal muscle protein extracts.

DISCUSSION

For investigation of newly discovered proteins, ones for which commercial antibodies are not available, it is helpful to produce specific antibodies as an alternative to tags and tag-specific antibodies. Here we present the production and analysis of three mouse polyclonal and one monoclonal antibody raised against the full-length protein, the N-terminus and, the C-terminus of the Ankrd2 protein. The choice of antigens was based on modular structure of the Ankrd2 protein, the N-terminus (1-120 aa), ankyrin repeats in the middle portion of the protein (121-278 aa), and the C-terminus (279-333 aa). Recombinant His-tagged proteins purified under denatured conditions were used for immunization of mice. Although all anti-Ankrd2 antibodies were generated against denatured antigens, they recognized denatured Ankrd2 in human heart and skeletal muscle extracts (Fig. 5), as well as native protein in muscle tissue (as demonstrated by immunohistochemistry) (Pallavicini et al., 2001). We experimentally confirmed the theoretical prediction of potential epitopes represented by amino acid sequences that may adopt an exposed conformation based on ProtScale analysis of Ankrd2 C-terminus. Using a panel of deletant mutants, we precisely mapped the epitope specifically recognized by the monoclonal anti-Ankrd2 antibody (clone 2F10) to the region of the last 10 aa of Ankrd2 (323-333 aa). This region may represent a linear epitope, to judge from its size. Epitope size is defined by the binding site of the antibody and limited by complementary binding between antigen and antibody. Epitopes can be linear or conformational. Typically, 6-7 amino acids or sugars can fit into the deep pocket structures of linear epitope binding sites; for example, the 6 His tag is only six amino acids but can be seen by its specific antibody. On the other hand, conformational epitopes of globular proteins cover much greater space on flatter surface binding sites of antibody and may consist of 15-22 amino acids (Kindt et al., 2006).

As the anti-N- and anti-C-Ankrd2 antibodies were made using the corresponding regions and a part of ankyrin repeats, we took into account the fact that the ankyrin repeats represent a common motif composed of 33 aa that mediate protein-protein interactions (Michaely and Bennett, 1992). We here confirm that the anti-N-terminal and anti-C-terminal Ankrd2 antibodies are specific for the N- and C-termini of Ankrd2 and do not recognize Ankrd2 ankyrin repeats; they are therefore unlikely to cross react with the large number of ankyrin repeat proteins in cells.

Western blot analysis of human heart and skeletal muscle proteins showed the existence of a single band that corresponds to the endogenous Ankrd2 protein, thus demonstrating the specificity of these antibodies raised against the human striated muscle protein Ankrd2. It has already been seen that Ankrd2 is more abundantly expressed in skeletal muscle than in the adult heart (Moriyama et al., 2001), as shown in Fig. 4. It would appear that the polyclonal anti-C-Ankrd2 antibody demonstrated the lowest affinity for endogenous Ankrd2.

In conclusion, we have characterized a set of highly specific and defined antibodies produced

in order to study a MARP protein family member, Ankrd2.

Acknowledgments — This work was supported by the Ministry of Science and Technological Development of Serbia (Project No. 143051). The NP1 cells used by us were a gift from Prof. G Valle (University of Padua, Italy), who originally obtained them from the late Prof. C Milstein. We thank G. Lunazzi and M. Sturnega (ICGEB, Trieste) for technical assistance in the immunization of animals used for antibody production and Dr. Dragica Radojković (IMGGE) for critical reading of the manuscript.

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КАРАКТЕРИЗАЦИЈА АНТИТЕЛА НА ХУМАНИ МИШИЋНИ ПРОТЕИН ANKRD2

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Протеин Ankrd2 поседује анкиринске поновке, специфично се експримира у скелетним мишићима и срцу и може бити локализован и у једру и у цитоплазми мишићне ћелије. Пошто антитела на овај протеин нису комерцијално доступна, у овом раду је описано генерисање и карактеризација три мишја поликлонска и једног моноклонског антитела добијених на цео, као и на амино-терминални и карбокси-терминални део протеина. Коришћењем рекомбинантних делетаната мапиран је епитоп моноклонског антитела величине 10 аминокиселина (323-333 ak), а такође је показано да поликлонска антитела на амино-терминални и карбокси-терминални део протеина специфично препознају Ankrd2, а не анкиринске поновке који су присутни у великом броју протеина. Сва четири антитела су високо специфична за ендогени Ankrd2 који се експримира у срцу и скелетним мишићима.