Production and Epitope Specificity of Monoclonal Antibody against Mouse Peptidylarginine Deiminase Type II

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Received June 16, 1994

Peptidylarginine deiminase catalyzes the conversion of arginyl residues in proteins to citrulline residues in the presence of Ca²⁺. We described the preparation of monoclonal antibody (subclass type IgG) specific to mouse peptidylarginine deiminase type II. The antibody had no effect on the enzyme activity and its specific epitope was localized in the eight-residue segment at the amino-terminal portion of the enzyme.

Peptidylarginine deiminase (protein-t-arginine inomohydrolase, EC 3.5.3.15) (PAD) catalyzes deamination of arginine residues of proteins to citrulline residues in the presence of calcium ion¹ and exists in various tissues of vertebrates.² ³ ⁴ Though the physiological role of the enzyme is not clearly understood, the findings that some structural proteins, such as myelin basic protein,⁵ ⁶ ⁷ fillagrin,⁸ and intermediate filament proteins⁹ ¹⁰ ¹¹ were degraded by PAD both in vivo and in vitro, suggest that the enzyme is involved in cell differentiation. We have reported that there are three types of PAD in mouse tissues and proposed designating them PAD types I, II, and III according to their elution order in anion-exchange column chromatography.¹² These enzymes showed similar catalytic properties, but their molecular weights, substrate specificities, and antigenic properties were different.¹³ ¹⁴ Among these variants, PAD type II has been the most studied. It is widely distributed in various kinds of tissues, such as brain, salivary gland, pancreas, skeletal muscle, and uterus¹⁵ and the enzymes purified from skeletal muscle were well characterized.¹⁶ ¹⁷ ¹⁸ ¹⁹ The amino acid sequences of PAD type II of rat¹³ ¹⁴ and mouse¹⁴ ¹⁷ have been deduced from their nucleotide sequences. The sequences of both enzymes were quite similar, although the murine enzyme has an additional eight residues at amino-terminus.¹⁸ ¹⁹ In addition, the z-amino group of the amino-terminal methionine residue of the murine enzyme was acetylated while that of the rat enzyme was not.¹⁸ ¹⁹ Here, we describe the preparation of monoclonal antibody (MAB) specific to mouse PAD type II and its specific epitope.

PAD type II was purified from mouse (ddy strain) skeletal muscle as described by Takahara et al.¹⁰ ¹¹ Production of MAb against the enzyme was done essentially according to the method of William and Alfred.¹⁵ In brief, mice (BALB/c) were pre-immunized by intraperitoneal injection of ddy mice spleen cells (10⁶ cells/body) with cyclophosphamide (0.1 mg/g of body weight). After two weeks, 100 μl of the enzyme solution (100 μg in phosphate-buffered saline) emulsified with an equal volume of Freund's complete adjuvant was injected into the mice. This immunization was repeated twice at two-week intervals (total of 2 times) and then the same amount of enzyme emulsified in incomplete adjuvant was injected as a booster. Spleen cells from the mouse were fused with myeloma cells (X63-Ag8-6.5.3.) to obtain hybridomas. Cell fusion, culture, and cloning of hybridomas were done by the method of Oi and Herzenberg.¹⁶

Using an enzyme-linked immunosorbent assay (ELISA),¹⁷ we obtained six hybridomas (EH2, EH7, DH7, DB8, DH11, EC11) that produced MAb against mouse PAD type II. The MAb of each hybridoma was prepared from ascitic fluids of BALB/c mice that had received intraperitoneal injections of the hybridoma cells. The MAb were purified with Anti-Gel Protein A MAPS II Kit (Bio-Rad). All of these MAb were found to belong to the same subclass type (IgG) and their specific binding activities to the enzyme were nearly the same (data not shown). These MAb reacted strongly with the intact enzyme (M₈ = 81 kDa) and their Western blotting patterns against several polypeptides derived from partial digestion with lysyl endopeptidase were very similar (data not shown). These results indicated that these MAb recognized an identical or very similar epitope on mouse PAD type II. Therefore, we examined in detail the properties of one of the MAb, MABEH7. Immunocross-activity test of MABEH7 to PAD type II from seven vertebrates (mouse, rat, rabbit, golden hamster, guinea pig, pig, and chicken) showed that the MAb reacted only with the mouse enzyme (data not shown). Our previous work with polyclonal antibodies against mouse PAD type II demonstrated that the enzymes from the rodsent share an antigenic determinant.¹⁰ These results show that this MAb strictly recognized a unique sequence on mouse PAD type II. Next, we examined the effects of MAb binding on enzyme activity. We had reported previously that when soybean trypsin inhibitor (Kunitz) was incubated with PAD type II, the trypsin-inhibitory activity of the inhibitor was lost completely. The inactivation of the inhibitor was due to the conversion of one arginine residue at the reactive site (Arg63) to a citrulline residue.¹⁸ MABEH7 had no effect on the activity of the enzyme up to a molar antibody:enzyme ratio of 10:1 (data not shown). Thus, the MAb probably binds an epitope on PAD type II that is distant from the active site of the enzyme. To identify the antigenic site of PAD type II that is recognized by MABEH7, the enzyme was reduced, S-pyridyl-ethylated and digested by lysyl endopeptidase. The digest was separated by reverse-phase HPLC and each peak was tested for immunoreactivity with the MAb by ELISA. One peptide (Peptide N) reacted with the MAb (data not shown). Through a combination of amino acid analyses and sequence data of mouse PAD type II,¹⁴ Peptide N was shown to correspond to the 46-residue amino terminal sequence (N³-actyl-MQPPIREMLRERTVR-LQYGRVEAVYVLQTLWDVYSAAPAGK) of mouse PAD type II.¹⁴ In an attempt to precisely locate the epitope, we cleaved Peptide N into smaller fragments with CNBr and purified the fragments by HPLC. A fixed amount of MABEH7 was mixed with various amounts of the peptide fragments and allowed to react with the enzyme bound to ELISA plates. The amino-terminal

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Abbreviations: PAD, peptidylarginine deiminase; MAB, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.
fragment (QPPIRENLM), at a molar ratio of 1:1, reduced the binding of MAbE7 to the enzyme by 50.3%, but the carboxy-terminal fragment (LRERTVRLQYGRVEAYVYVLTQLWDVVSAPAGAK) did not interfere with the binding (Fig.). These results indicate that the specific epitope of the MAbs is in the eight-residue segment at the amino-terminal portion of the enzyme.

The MAbs described here will be useful in further studies of type physiological role of PAD type II in the mouse. Studies aimed at using the MAbs for purification and immobilization of the enzyme are in progress.

References