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Efficient Degradation *in vitro* of All Intermediate Filament Subunit Proteins by the Ca^{2+}-Activated Neutral Thiol Proteinase from Ehrlich Ascites Tumor Cells and Porcine Kidney

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Vimentin, desmin, glial fibrillary acidic protein, neurofilament triplet proteins, and a mixture of cytokeratins were digested with Ca^{2+}-activated neutral thiol proteinase isolated from Ehrlich ascites tumor (EAT) cells and porcine kidney. All intermediate filament proteins were degraded by the proteinase, although with different rates and Ca^{2+} optima. These results are in part at variance with our previous statement that the Ca^{2+}-activated proteinase from EAT cells is specific for vimentin and desmin.

INTRODUCTION

Ca^{2+}-activated neutral thiol proteinases are non-lysosomal, cytosolic enzymes with a wide distribution among vertebrates (Dayton *et al.*, 1976; Ishiura *et al.*, 1978; Mellgren, 1980; Murachi *et al.*, 1981; Zimmerman and Schlaepfer, 1982; Nelson and Traub, 1982a, b). Dependent on the Ca^{2+} requirement, two different forms of enzyme, activatable at μM and mM Ca^{2+} concentrations, respectively, can be distinguished (Mellgren, 1980; Murachi *et al.*, 1981; Dayton *et al.*, 1981; Inomata *et al.*, 1984;

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Zimmerman and Schlaepfer, 1984). Although little is known about the physiological relationship between the two enzyme forms, it seems to be clear that both types are regulated by transient and local increases in the intracellular concentration of free Ca^{2+}. With respect to substrate specificity, Ca^{2+}-activated neutral proteinases appear to be involved in the turnover of myofibrillar proteins (Dayton *et al.*, 1976; Dayton *et al.*, 1981; Reddy *et al.*, 1975; Sugita *et al.*, 1984), in the degradation of constituents of the cyto- (Zimmerman and Schlaepfer, 1982; Nelson and Traub, 1982a, b; Dayton *et al.*, 1981; Zimmerman and Schlaepfer, 1984; Sugita *et al.*, 1984; Nelson and Traub, 1983; Sandoval and Weber, 1978; Yoshimura *et al.*, 1984) and membrane (Thomas *et al.*, 1983; Pant *et al.*, 1983; Lorand *et al.*, 1983; Murakami *et al.*, 1981) skeleton, in the processing of steroid (Puca *et al.*, 1977; Vedeckis *et al.*, 1980) and membrane (O'Connor-McCourt and Hollenberg, 1983; Baudry and Lynch, 1980) receptors and in enzyme activation (Inoue *et al.*, 1977; Huston and Krebs, 1968).

We have recently reported on the isolation and characterization of a Ca^{2+}-activated proteinase from cultured EAT cells (Nelson and Traub, 1981, 1982a) and stated that the enzyme has specificity for the intermediate filament (IF) proteins, vimentin and desmin. In the following, we were successful in isolating another such enzyme from porcine kidney that in its physicochemical and functional properties is identical to the enzyme prepared from EAT cells (Traub, 1984). With larger quantities of both pure enzyme and pure substrate proteins at our disposal, we have reinvestigated the substrate specificity of the Ca^{2+}-activated proteinase. Here, we show that the enzyme has a lower specificity than hitherto presumed in that it processes not only vimentin and desmin but all IF subunit proteins, although with different rates and at different Ca^{2+} optima.

MATERIALS AND METHODS

Vimentin was isolated from cultured EAT cells (Nelson and Traub, 1982c; Nelson *et al.*, 1982), desmin from porcine stomach smooth muscle (Vorgias and Traub, 1983a), glial fibrillary acidic protein (GFAP) from bovine brain white matter (Vorgias and Traub, 1983b), neurofilament proteins (NFPs) from porcine spinal cord (Traub *et al.*, 1985), cytokeratins from newborn rat skin (Yen *et al.*, 1980), and Ca^{2+}-activated proteinase from porcine kidney (Traub, 1984) and EAT cells (Nelson and Traub, 1982a; Traub, 1984).

The digestion of IF proteins with Ca^{2+}-activated proteinase was carried out in 200 μl 10 m*M* Tris acetate, pH 7.6, 150 m*M* KCl, 0 to 2 m*M* Ca^{2+}, 6 m*M* 2-mercaptoethanol, employing 60 μg substrate and 2.5 μg enzyme. After incubation at 37°C for various lengths of time, the reactions were stopped either by (1) precipitation of protein with trichloroacetic acid at a final concentration of 5%, (2) addition of excess EDTA, or (3) addition of 5 × SDS-sample buffer. Modifications of the standard enzyme assay are indicated in the legends to the figures of the "Results and Discussion" section.

Polyacrylamide gradient slab gel electrophoresis in the presence of SDS (Egberts *et al.*, 1976) and in urea/acetic acid as buffer system (Traub and Boeckmann, 1978) was performed as described previously.

RESULTS AND DISCUSSION

The purpose of the present investigation was to further characterize the Ca^{2+}-activated neutral thiol proteinase from EAT cells (Nelson and Traub, 1982a; Traub, 1984) and porcine kidney (Traub, 1984) with respect to its possible specificity for certain IF proteins. First, we followed the kinetics of the degradation of vimentin, desmin, GFAP and NFP68 at 37°C in the presence of 150 m*M* KCl and 0.5 m*M* Ca^{2+}; the Ca^{2+} concentration was clearly above the threshold values characteristic of the individual IF proteins. Employing the Lineweaver–Burk double reciprocal plot (Fig. 1), the Henri–Michaelis–Menten constants were calculated to be 1.54×10^{-7} *M* for vimentin, 2.94×10^{-7} *M* for desmin, 6.58×10^{-7} *M* for GFAP, and 6.7×10^{-7} *M* for NFP68. These values show that among the IF proteins tested vimentin is the best

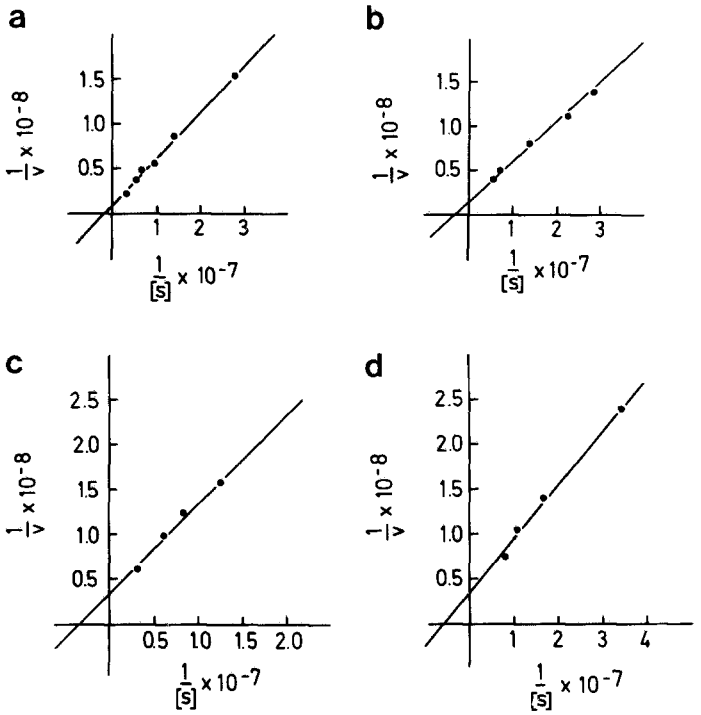

Fig. 1. Lineweaver–Burk plots of the degradation of vimentin (panel a), desmin (panel b), GFAP (panel c), and NFP68 (panel d) by the Ca^{2+}-activated proteinase from porcine kidney. The initial rates of IF protein degradation were determined by digesting 1 to 8 μg protein at an enzyme concentration of 2×10^{-9} *M* in 200 μl of 10 m*M* Tris acetate, pH 7.6, 150 m*M* KCl, 0.5 m*M* Ca^{2+}, 6 m*M* 2-mercaptoethanol for 5 min at 37°C. The reactions were stopped by the addition of 200 μl 10% trichloroacetic acid and the amount of remaining protein was determined by polyacrylamide gel electrophoresis in urea-acetic acid as buffer system. The gels were stained with Coomassie Brilliant Blue and scanned at 590 nm. The rate v of protein degradation was defined as moles of protein digested per min employing appropriate protein calibration curves. The substrate concentration [S] was defined as moles of initial filament protein per reaction mixture.

substrate of the Ca^{2+}-activated proteinase from EAT cells or porcine kidney. They are slightly at variance with data published previously on the proteolysis of vimentin and desmin (Nelson and Traub, 1982a). The deviations are probably due to differences in the methodology used for the isolation and purification of enzyme and substrate proteins. Since our first reports (Nelson and Traub, 1981, 1982a, 1983), we repeatedly observed variations in the degradability of IF proteins by Ca^{2+}-activated proteinase as a result of different physical and chemical treatments of the proteins.

Figure 2 shows the SDS-electrophoresis profiles of the degradation products obtained from different IF proteins at various Ca^{2+} concentrations. It was particularly characteristic of the non-neuronal IF proteins that their digestion led to the formation of two sets of breakdown products with a molecular weight gap of 10,000 to 13,000 (Fig. 2a–c). While the larger digestion products presumably still contain an intact α-helical rod domain, those of the lower molecular weight group might have arisen from cleavage of the non-α-helical spacer sequences of the rod domains. It is striking that even in the presence of excess Ca^{2+}, and with the employment of longer digestion times (data not shown), the further degradation of the high-molecular-weight polypeptides did not attain completion. The reason for this premature cessation of the degradation processes is not known. It is, however, not due to improverishment of the reaction mixtures of Ca^{2+}-activated proteinase. Although its large catalytic subunit recognizable in the gel profiles as a faint, sharp band on top of the IF protein bands to some extent underwent autodigestion, even at higher Ca^{2+} concentrations enough enzyme was left for continuing proteolysis.

In comparison with non-neuronal IF proteins, the neurofilament triplet proteins showed a somewhat different response to incubation with Ca^{2+}-activated proteinase, with respect to both Ca^{2+} requirement and peptide pattern produced (Fig. 2d–f). As already indicated in the case of GFAP (Fig. 2c), they needed higher Ca^{2+} concentrations for efficient degradation. While vimentin and desmin were rapidly degraded at 0.3 to 0.4 m*M* Ca^{2+} (Fig. 2a, b), GFAP, NFP68, and NFP145 reacted only reluctantly at this Ca^{2+} concentration. The breakdown of NFP200 required at least 0.8 m*M* Ca^{2+} (Fig. 2f).

Concerning the electrophoresis profiles of the breakdown products derived from neurofilament proteins, only that obtained from NFP68 (Fig. 2d) was similar to those characteristic of non-neuronal IF proteins (Fig. 2a–c). The digestion of NFP145 produced a collection of low- and high-molecular-weight polypeptides among which two breakdown products with approximate molecular weights of 95,000 and 105,000 were most prominent (Fig. 2e). From NFP200, preferentially large digestion products were obtained with molecular weights in the 165,000 to 185,000 range (Fig. 2f). It remains to be elucidated whether this peculiar behavior of the two larger neurofilament proteins is caused by their unusually long, non-α-helical C-terminal extensions (Geisler *et al.*, 1984; Geisler *et al.*, 1985).

The digestion products of the various IF subunit proteins have also been subjected to 2D-polyacrylamide gel electrophoresis (data not shown). In all cases examined, including the large neurofilament proteins, the high-molecular-weight polypeptides appearing first during digestion had decreasing isoelectric points and were arranged in a staircase-type pattern indicating that the proteinase removes small basic peptides from the non-α-helical ends of the IF protein molecules. Since only the N-terminus of

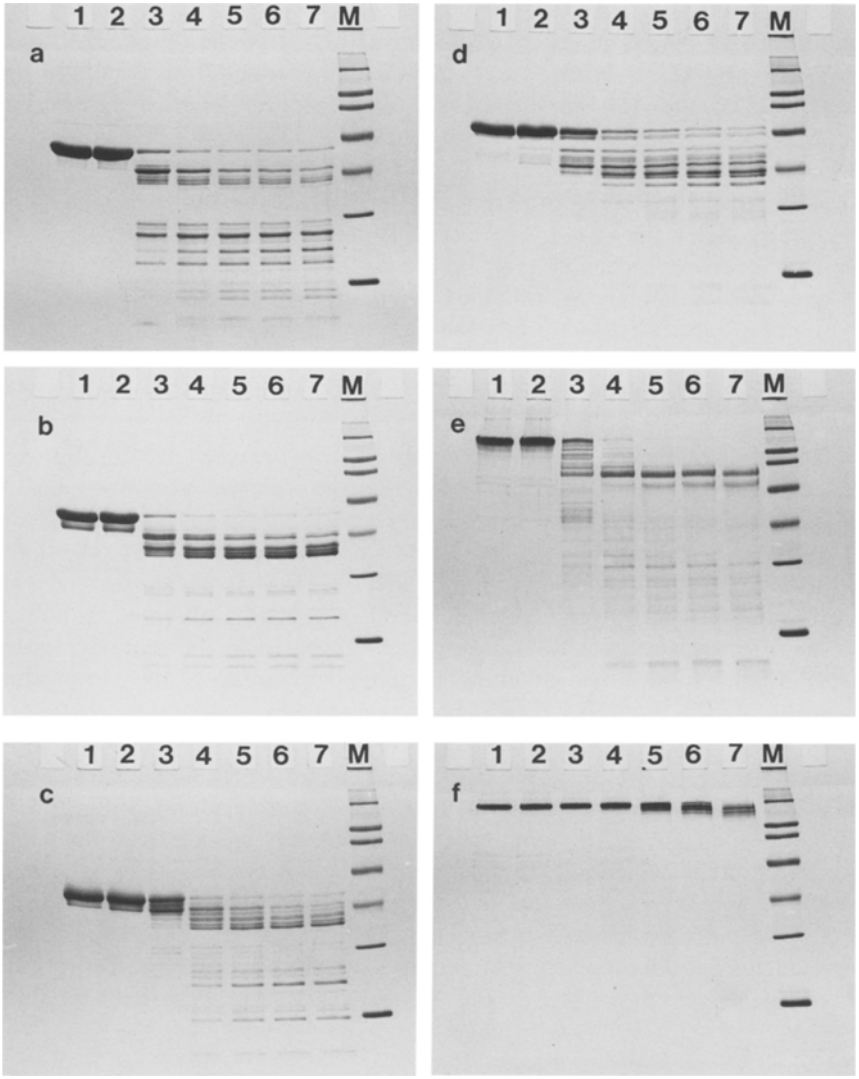

Fig. 2. SDS-polyacrylamide gel electrophoretic presentation of the digestion of vimentin (panel a), desmin (panel b), GFAP (panel c), NFP68 (panel d), NFP145 (panel e), and NFP200 (panel f) by the Ca^{2+}-activated proteinase from porcine kidney at different Ca^{2+} concentrations. Standard reaction mixtures containing 150 m*M* KCl and (lanes 1 to 7) 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 m*M* Ca^{2+} were incubated at 37°C for 5 min. The reactions were stopped by the addition of 20 μl 5 × SDS-sample buffer to each 200 μl incubation mixture and 7 μg peptide material was applied to the gel. Lane M: molecular weight markers (from the top to the bottom: myosin, 205 kD; β-galactosidase, 116 kD; phosphorylase a, 92.5 kD; bovine serum albumin, 68 kD; ovalbumin, 45 kD; carboanhydrase, 31.5 kD; myoglobin, 17.8 kD.)

these proteins is rich in arginine and poor in acidic amino acid residues (Geisler *et al.*, 1982; Geisler and Weber, 1982; Geisler *et al.*, 1983a; Geisler *et al.*, 1983b; Quax *et al.*, 1983; Geisler *et al.*, 1984; Geisler *et al.*, 1985; Balcarek and Cowan, 1985), it is very likely this end of the protein molecules which is preferentially attacked by the proteinase

(Nelson and Traub, 1983). Allowing for the additional fact that the sizes of the smallest polypeptides of the high-molecular-weight group of degradation products (Fig. 2a–c) are close to that of the α-helical, 38 kD rod domain generated from desmin by limited chymotryptic digestion (Geisler *et al.*, 1982), the present results are in agreement with the general concept of IF protein structure (Geisler *et al.*, 1983a; Quax *et al.*, 1983; Geisler and Weber, 1983).

Finally, the cytokeratins from newborn rat skin have also been included in the present study. Since they were only available as a relatively crude mixture, only a preliminary characterization of their susceptibilities to Ca^{2+}-dependent proteolysis could be carried out. Figure 3 shows the Ca^{2+} dependency of the degradation process. All cytokeratin species proved to be good substrates of the Ca^{2+}-activated proteinase from porcine kidney, although the Ca^{2+} requirement was substantially higher than in the case of vimentin, desmin, and GFAP (Fig. 2a–c). Approximately 1 m*M* Ca^{2+} was needed for efficient breakdown of the cytokeratin proteins.

The electrophoresis profiles shown in Fig. 2a–d are the result of 5 min digestions of the individual IF proteins. To demonstrate that the reactions were not prematurely terminated, long-term kinetics of degradation were followed at 150 m*M* KCl and 0.4 and 2 m*M* Ca^{2+}, respectively. While 0.4 m*M* Ca^{2+} was slightly above the threshold concentration required for the digestion of NFP68, 2 m*M* Ca^{2+} represented a strong excess of Ca^{2+} ions. However, no significant differences were observed; at both Ca^{2+} concentrations the same degradation products appeared in the same chronological sequence. Figure 4 presents a gel electrophoretic comparison of the end products of degradation of all non-epithelial IF proteins examined.

The reason why neurofilament proteins and cytokeratins have higher Ca^{2+} requirements for degradation than vimentin and desmin is not known. Since the proteinase is fully active at substantially lower Ca^{2+} concentrations, additional Ca^{2+} might be needed to induce configurational changes in the neurofilament and cytokeratin proteins which might render them more susceptible to proteolytic attack. However, it is also conceivable that *in vivo* the various IF subunit proteins characteristic of certain cell types are processed by specific Ca^{2+}-activated proteinases.

In conclusion, the present investigation has shown that, in contrast to previous reports from our laboratory (Nelson and Traub, 1981, 1982a, 1983), the Ca^{2+}-activated

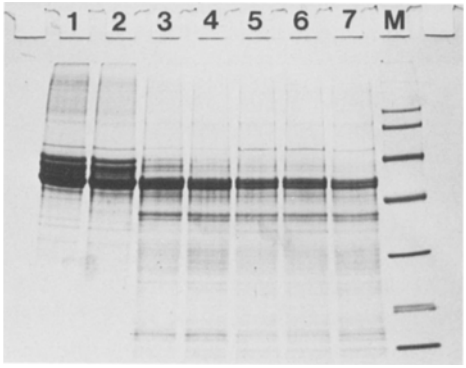

Fig. 3. Ca^{2+}-dependence of the degradation of cytokeratins from newborn rat skin by the Ca^{2+}-activated proteinase from porcine kidney. Standard reaction mixtures containing 150 m*M* KCl and (lanes 1 to 7) 0, 0.5, 1, 1.5, 2, 3, 4 m*M* Ca^{2+} were treated in the same way as specified in the legend to Fig. 2. Lane M: molecular weight markers (see Fig. 2).

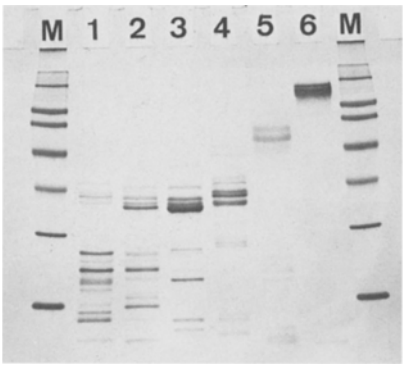

Fig. 4. SDS-polyacrylamide gel electrophoretic comparison of the end products of the digestion of non-epithelial IF proteins by the Ca^{2+}-activated proteinase from porcine kidney. Vimentin (lane 1), GFAP (lane 2), desmin (lane 3), NFP68 (lane 4), NFP145 (lane 5), NFP200 (lane 6) were digested for 30 min at 37°C in standard reaction mixtures containing 150 m*M* KCl and 2 m*M* Ca^{2+}. The individual samples were analysed as described in the legend to Fig. 2. Lanes M: molecular weight markers (see Fig. 2).

neutral thiol proteinase isolated from EAT cells and procine kidney degrades not only vimentin and desmin but also GFAP, the neurofilament triplet proteins and even cytokeratins with high efficiency. However, the various IF subunit proteins show different sensitivities to the proteinase, in regard to both Ca^{2+} requirement and rate of degradation. Whether these differences provide the basis for proteinase specificity and regulation of IF protein turnover *in vivo* will be the subject of future research.

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