Monoclonal antibodies block the bromelain-mediated release of human placental alkaline phosphatase from cultured cancer cells

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Certain monoclonal antibodies (mAbs) to human placental alkaline phosphatase (PLAP) block bromelain cleavage of a 2-kDa segment from each of the two polypeptide chains of PLAP. These mAbs also prevent the release of PLAP from cultured cancer cell surfaces by bromelain. Such proteolysis-blocking mAbs serve as tools to specifically modify the molecular topography of cell surfaces by protease treatment.

Alkaline phosphatase *Blocking monoclonal antibody* *Bromelain* *Proteolysis*

1. INTRODUCTION

Recently we reported that 3 out of a total of 11 mAbs to human placental alkaline phosphatase (PLAP) blocked the cleavage by trypsin of a 10-kDa segment from PLAP [1]. We proposed that similar blocking antibodies to other antigens might be useful for proteolysis of the native molecule to polypeptides having desired lengths and for studies of the effects of proteolytic processing on the biological activity of a protein. The blocking functions of mAbs also serve as a means to relate the spatial arrangements of the antigenic sites on the native molecule.

Now we extend that list to include the use of mAbs to block the release of proteins from the cell surface by proteolytic treatment. Several mAbs specific for PLAP have such an activity. They block the bromelain proteolytic cleavage of a 2-kDa segment from both polypeptide chains of PLAP and also block the bromelain-mediated release of the protein from cell surfaces. Thus, these mAbs serve as a means to prepare modified cells differing in their expression of cell-surface PLAP. Such a model system is useful for investigations of the biological role of PLAP, a function that has thus far not been defined. By using proteolysis blocking mAbs to other antigens and appropriate proteases and lipases, this approach may be extended to studies of the biological functions of a variety of cell-surface molecules.

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2. MATERIALS AND METHODS

2.1. *Production of mAbs*

Eleven mAbs to PLAP were previously prepared and described [1,2]. All mAbs employed in the present study were either IgG1, IgG2a, or IgG2b [1].

2.2. *Bromelain cleavage of PLAP*

PLAP (S-variant) was purified from an individual placenta as previously described [3]. The protein was labeled with ^{125}iodine by the method of Hunter and Greenwood [4]. Bovine serum albumin (100 μg) was used as a carrier molecule in the proteolytic digestions of ^{125}I-PLAP (50 ng). In some samples the effects of various mAbs (10 μg) on proteolysis were examined. The protein mixtures were incubated with 10% bromelain (Sigma,

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St. Louis, MO) for 30 min at 37°C, pH 6.8 in 50 mM Hepes. Immediately after proteolysis, the samples were mixed with an equal volume of 10% 2-mercaptoethanol and 2% SDS and boiled for 3 min for evaluation in SDS–PAGE.

2.3. *Release of PLAP from the cell surface by bromelain*

Cells of the HeLa TCRC-1 adenocarcinoma line (10^5) grown on 24-well tissue culture plates were incubated either with or without 1.0 ml mAb (50 μg/ml) in 60 ml 25 mM Hepes-buffered saline (pH 6.8) for 30 min at 4°C. Bromelain (100 μg/ml) was added to each well of the plate and further incubated for 30 min at 37°C. After proteolysis, the cells were no longer attached to the plate and were removed using a pipette. The cells were centrifuged, the pellet and supernatants separated, and both immediately assayed for PLAP catalytic activity. Substrate, *p*-nitrophenyl phosphate (10 μg/ml) at pH 9.8 in 0.1 M sodium bicarbonate buffer was added to the pellet and to an aliquot of the supernatant and the optical densities at 405 nm were determined after 30 min incubations.

2.4. *Observation of membrane proteins released from carcinoma cells by bromelain*

HeLa TCRC-1 cells (10^7) were radiolabeled using lactoperoxidase [5]. They were distributed into fractions and preincubated with either a nonspecific IgG1 or PLAP-specific mAbs (50 μg/ml). The cells were then treated with bromelain (1 mg/ml) in 50 mM Hepes–saline, pH 6.8, and the liberated and cell-bound components were separated by centrifugation. Each fraction was dissolved in 5% 2-mercaptoethanol and 1% SDS and boiled for 3 min prior to separation of components in SDS–PAGE.

2.5. *Immunoprecipitation of PLAP*

Immunoprecipitation of the supernatants following centrifugation of bromelain-proteolysed cells was performed using mAb-coated beads. Each supernatant (100 μl) was incubated with 50 μl of mAb F11 bound to sheep anti-mouse IgG beads in phosphate-buffered saline containing 5% bovine serum and 0.1% Tween-20. Sheep antibodies were covalently attached to methylacrylate beads (Dynospheres, Dyno Industrier A/S, Oslo). The beads were washed 3 times in phosphate-buffered saline and bound proteins were then denatured in SDS and 2-mercaptoethanol as described above prior to electrophoresis.

2.6. *SDS–PAGE*

SDS–PAGE in 10% polyacrylamide slab gels was performed as described by Laemmli [6]. After Coomassie blue staining to detect the standard M_r markers, the gels were dried and subjected to autoradiography.

3. RESULTS

3.1. *Monoclonal antibodies block bromelain cleavage of PLAP*

In a previous study we compared the specificities of two sets of mAbs produced against two different immunizing forms of PLAP [7], either purified PLAP or whole HeLa TCRC-1 cells which express the enzyme on their surface [8]. We found that two out of four antibodies to purified PLAP (H7 and B10) bound to a region of the molecule that apparently was not immunogenic when the immune system was presented with PLAP bound to HeLa TCRC-1 cells. Furthermore, these two mAbs bound poorly to the membranous form of the enzyme. However, by solubilizing the membrane with nonionic detergent or by releasing the enzyme from the cell surface using bromelain, the antigenic site for these two mAbs was exposed and became fully immunoreactive [7].

These results implied that since these mAbs (H7 and B10) appeared to bind at a site on PLAP hidden in part by membrane components, they might also interfere with the proteolytic action of bromelain which cleaves at a site on the molecule near the membrane and releases PLAP from the cell [9]. In initial experiments we incubated ^{125}I-labeled PLAP with 11 mAbs to the antigen and examined their ability to block cleavage by bromelain. The results are shown in fig.1. Normally, bromelain cleaves a 2-kDa segment from PLAP [10]. In the presence of the mAbs B10 and H7 this cleavage is blocked. Partial effects are observed with F11 and C4, while the remaining 7 mAbs appear to have no blocking activity. This experiment thus defines a second proteolytic site on PLAP distinct from the trypsin cleavage site previously reported that can be blocked by mAbs [1]. It is interesting to note that while H7 completely protects

Fig.1. Effects of mAbs on bromelain cleavage of PLAP. PLAP (^{125}I-labeled) was incubated with the various mAbs, proteolyzed with bromelain, and electrophoresed in SDS–PAGE. Although not shown in this photograph PLAP migrates to the same position as the single band shown with B10.

the bromelain cleavage site, it partially blocks the trypsin cleavage site. Similarly, C4, which completely blocks the trypsin cleavage site, partially protects the bromelain cleavage site. The overlapping effects observed with these two mAbs may be due to the folding of the polypeptide chain that positions these two cleavage sites near to one another in the 3-dimensional structure. This concept is further described in section 4.

3.2. *Monoclonal antibodies block the release of PLAP from the cell membrane by the protease bromelain*

The blocking effects of the mAbs observed with purified PLAP described above are reproduced when HeLa TCRC-1 cells are treated with bromelain as shown in fig.2. B10 is the most effective blocking antibody, allowing for the retention of over 90% of the PLAP enzyme activity on the cells during bromelain treatment. Most of the

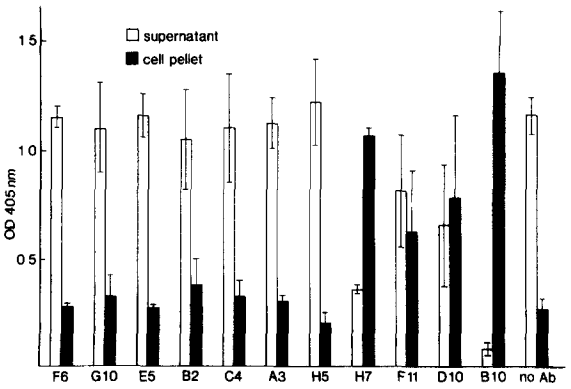

Fig.2. Effects of mAbs on the release of PLAP from the surface of HeLa TCRC-1 adenocarcinoma cells by bromelain. PLAP catalytic activity was monitored immediately following proteolysis using *p*-nitrophenylphosphate as substrate.

other antibodies, G10, B2, E5, F6, H5 and C4, have no blocking activity. Partial blocking activity is observed with H7 and F11 as expected from the results shown in fig.1, and also by D10. The latter antibody does not block bromelain cleavage of purified PLAP. Furthermore, C4, which appears to partially block the cleavage of the free enzyme, has no effect on membrane-bound PLAP. The differences observed with these two mAbs, D10 and C4, may be due to interference by other molecules in the membrane. Since the most complete blocking effect by the mAbs was observed with B10, the effects of that mAb were explored further.

3.3. *Selective retention of PLAP on the plasma membrane during proteolysis using a blocking monoclonal antibody*

To examine molecular profiles of the cell surface of HeLa TCRC-1 cells before and after bromelain treatment, the cells were surface labeled with ^{125}iodine employing lactoperoxidase [5]. After they were radiolabeled, the cells were incubated either with a nonspecific antibody, F6 which does not block bromelain proteolysis, or B10 which does. The cells were then incubated with bromelain for 30 min and centrifuged. The supernatants and cell pellets were electrophoresed in SDS–PAGE under reducing and denaturing conditions. The results are shown in fig.3.

The prominent band at 67 kDa (fig.3) which is present on the B10 preincubated cells is absent in the cell pellet of the other two incubations. Concordantly in the supernatants of the latter there appears a band at 65 kDa that is absent from the supernatant of the B10 preincubated cells. The 65-kDa band is proteolysed PLAP resulting from bromelain cleavage of a 2-kDa segment from both polypeptide chains [9]. This is confirmed by immunoprecipitation of proteins in the three supernatants using mAb F11 coupled to sheep anti-mouse IgG on Dynosphere monodisperse polymer particles (Dyno Industrier A/S, Oslo) (fig.4). This antibody binds at a site distinct from the sites for F6 and B10 and, therefore, can bind simultaneously to the molecule [2,7]. No precipitable material is observed in the B10 preincubated material indicating that PLAP was not liberated from the cell surface in that case.

Other than the 67- and 65-kDa bands of PLAP, no apparent differences are observed among the

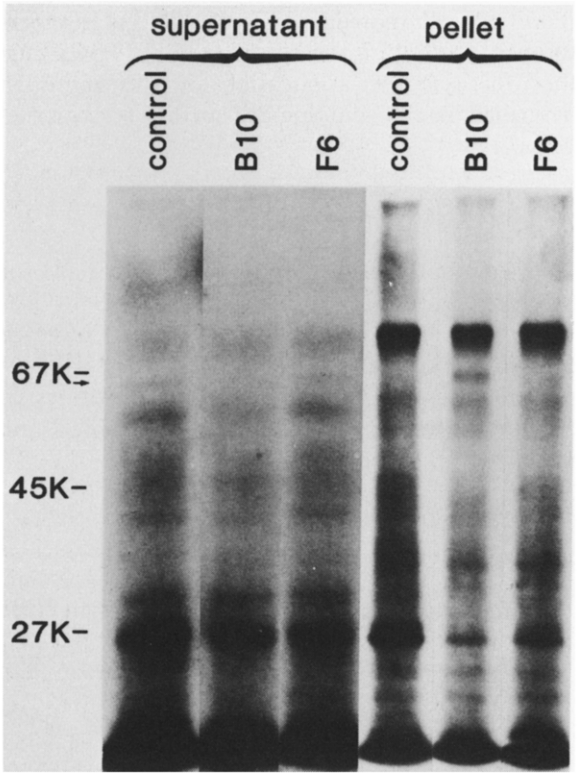

Fig.3. Autoradiograph of SDS–PAGE of iodinated cell surface components in the supernatants and the cell pellets of mAb-pretreated, bromelain proteolyzed cells. The control mAb used was nonspecific IgG1.

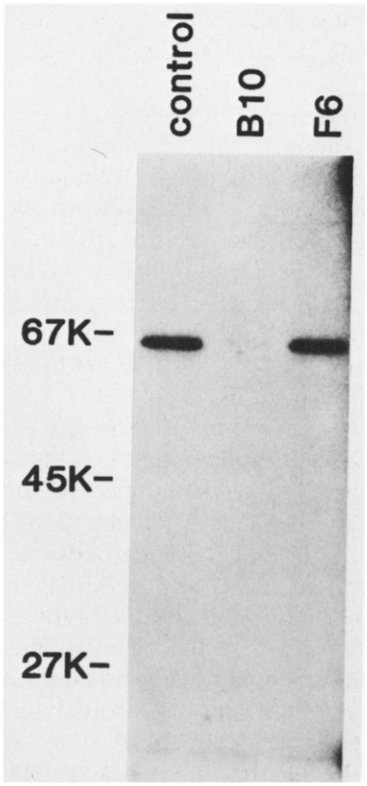

Fig.4. Immunoprecipitation of the supernatants from bromelain proteolyzed, iodinated HeLa TCRC-1 cells using mAb F11 and sheep antibodies to mouse IgG covalently attached to methylacrylate beads. The cells were preincubated with the indicated mAbs prior to proteolysis.

three preparations, indicating that the modified cells in each experiment are otherwise identical. It is interesting to note that not all of the radiolabeled proteins are liberated from the membrane using bromelain.

The removal of PLAP from the membrane by bromelain is not permanent. Previously it was shown that within 55 min after proteolysis, PLAP begins to reappear at the cell surface [10]. Thus, for use in the investigation of the biological role of PLAP, the modified cells must be used immediately after preparation. Over 90% of the treated cells failed to absorb trypan blue, indicating that they were largely impermeable to molecules not actively transported into the cells. Therefore, since many of the cell surface proteins remain on the membrane after bromelain proteolysis (fig.3), it is likely that these modified cells will retain many of the biological activities of the intact cell. Furthermore, most of the cellular architecture necessary for those functions is likely to remain intact or at least be more similar to the native state than that observed in reconstituted vesicles.

4. DISCUSSION

In a previous report we described a set of mAbs to PLAP (C4, E5, and F6) that protected the only trypsin cleavage site on each of the two PLAP polypeptide chains of the native protein [1]. The present study identifies a different proteolytic cleavage site on each polypeptide chain recognized by bromelain that can be protected by a second set of mAbs (B10 and H7).

It has been suggested from tandem proteolytic digests of trypsin and bromelain that these two

proteases recognize sites at opposite ends of the polypeptide chain [11]. The fact that each of two different sets of mAbs recognizing distinct epitopes [7] protect different sites from proteolytic cleavage supports this conclusion. It is possible that the amino and carboxyl terminal ends of each polypeptide chain are folded near to one another in the 3-dimensional structure of PLAP, as is the case with *E. coli* alkaline phosphatase [12]. This would explain the partially overlapping proteolysis-blocking effects on both trypsin and bromelain cleavage observed with the mAbs C4 and H7 described in section 3.

Of potential importance to cell-surface biochemistry is the description in this report of a novel means to engineer specific topographical changes on cells using mAbs that block proteolysis. In our model system, cultured cancer cells were modified such that they either did not have PLAP on their surface, or they expressed that enzyme with an attached monoclonal antibody. Bromelain was used as the protease to remove cell surface proteins and its proteolytic attack on PLAP was specifically blocked by employing a mAb to PLAP that binds near to the bromelain cleavage site.

This approach may have an advantage over traditional cell models where membrane components are reconstituted into membrane vesicles. The removal of molecules from the cell by proteases is not likely to alter the relative orientations of remaining components left on the membrane. Thus, interactions that are required for the biological activity of a molecule are more likely to occur in this model system than in reconstituted vesicles.

It is likely that antibodies similar to those presented here which block bromelain proteolysis of other cell surface proteins can be prepared. Furthermore, other enzymes that remove molecules specifically from the cell surface also exist. For example, phosphoinositol-specific phospholipase C has been shown to release alkaline phosphatases from cells [13,14]. If mAbs block the action of other proteases or lipases, each of which releases different components from the membrane, it will be possible to prepare a variety of short-term modified cells having different molecular repertoires expressed on their surface. This would further increase the application of the approach described here in studying cell-surface macromolecules.

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