The 175 Antigen Expressed on Myeloid and Erythroid Cells During Differentiation Is Associated With Serine Protease Activity

By David Deane, Lesley Inglis, and David Haig

Monoclonal antibody 175 recognizes a cell-surface antigen on more than 80% of nucleated ovine bone marrow cells (BMC). The distribution is unusual, as the majority of differentiated myeloid and erythroid cells express the antigen (175 antigen), whereas mast cells, basophils, and the majority of lymphocytes do not. The level of 175 antigen expression has been shown to increase as BMC differentiate during hematopoiesis. Previous attempts to identify the 175 antigen have been unsuccessful. In this study, the 175 antigen was affinity-purified and shown to contain serine protease activity. Immunoblot analysis following sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of bone marrow cell lysates run under reducing or nonreducing conditions showed two closely adjacent protein bands (a doublet) of 28 to 30 kD molecular weight. N-linked deglycosylation showed that the 30-kD band was a glycosylated form of the 28-kD protein. Both protein bands shared the same N-terminal amino acid sequence over 20 residues, with high homology with serine proteases. Affinity-purified 175 antigen was proteolytic in substrate gels, the activity being inhibited by the 175 monoclonal antibody (Mab) and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), but not by metallo, thiol, or acid protease-specific inhibitors. The 175 antigen is therefore part of a growing family of cell-surface proteases associated with hematopoietic cell differentiation.

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end of this period, a further 1 U of enzyme was added and the incubation repeated.

**N-terminal sequence analysis.** Affinity-purified 175 antigen, separated by SDS-PAGE, was transferred by the semidyry method to an inert support material, Immobilon-P (Millipore, Watford, UK) in 10 mmol/L of 3-[cyclohexylaminol]-propane-sulfonic acid (CAPS; Sigma). Protein was stained with 0.25% Coomassie brilliant blue in 50% methanol and 10% acetic acid fixative, and sequenced by the Microchemistry Department at the AFRC Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham, Cambridgeshire, UK.

**Radioiodination of cell-surface proteins.** BMC surface proteins were radiolabeled with I²¹² using the lactoperoxidase method of Marchalonis. Briefly, 8 x 10⁶ freshly isolated BMC were resuspended in 0.5 mL of PBS containing 9.1 U of lactoperoxidase (Boehringer Mannheim) and 500 μCi of carrier-free NaI²¹² (Amersham). The reaction was initiated by the addition of 2 μL of 0.0015% freshly diluted H₂O₂. Three further additions of H₂O₂ were made every minute before the reaction was terminated by the addition of PBS containing 1 mmol/L of dithiothreitol and 5 mmol/L of tyrosine (Sigma). The cells were then washed extensively in PBS and split into two aliquots to which was added 500 μL of PBS containing 2% bovine serum albumin (BSA) and 0.05% sodium azide, then either 5 μg of 175 MoAb or 5 μg of the IgM fraction of VPM12 for 40 minutes on ice. Cells were then washed twice in PBS and lysed as described previously in 1 mL of lysis buffer. Aliquots of 100 μL (containing ~15 μCi of radiolabeled product) of each lysate were added in quadruplicate to wells of a 96-well enzyme-linked immunosorbent assay plate (Dynatech Laboratories, Billeighurst, UK) coated with 1 μg/mL of sheep antimouse IgM antibody. Non-specific binding of protein was blocked by washing the plates with 3% BSA in PBS. After washing the wells extensively, bound complex was removed by the addition of 100 μL of SDS-PAGE sample buffer. Washed material was concentrated 10-fold in concentrator cells and analyzed by SDS-PAGE on a 4%, 10% discontinuous gel system run under reducing conditions as described previously.

**Inhibition of protease activity by specific protease inhibitors.** Affinity-purified 28-kD 175 antigen in 30-μg aliquots was incubated with the following previously optimized concentrations of specific protease inhibitors in 0.1 mL of 25-mmol/L Tris-buffered saline at pH 8.0 for 30 minutes at room temperature: 2 mmol/L phenylmethylsulfonyl fluoride (PMSF; Boehringer Mannheim; inhibits serine proteases), 100 μmol/L 1-trans-epoxysoycinnic acid (E-64; Boehringer Mannheim; inhibits thiolproteases), 1 mmol/L 1,10-phenanthroline (Sigma; inhibits metalloproteases), and 50 μmol/L pepstatin A (Sigma; inhibits acid proteases). To each mixture was then added an equal volume of SDS-PAGE sample buffer without 2-mercaptoethanol and without boiling, and samples were run on 4%, 10% gels as described earlier but with 0.2% (wt/vol) azocasein (Sigma) in the separating gel component. Once run, substrate gels were washed in 2 vol of 2.5% Triton X100 in water for 2 hours, then briefly in water and finally in PBS before an overnight incubation at 37°C. Digestion of the substrate was detected by staining the gel with Coomassie brilliant blue and identifying clear areas.

**Inhibition of protease activity by 175 MoAb.** Affinity-purified 175 antigen was separated on substrate gels containing azocasein as described, along with either a 1-mg/mL gel of 175 MoAb or VPM12 MoAb. In adjacent tracks, 20- to 50-μg samples of the proteases trypsin, chymotrypsin (Sigma), and elastase (Boehringer Mannheim) were also run.

**RESULTS**

**Analysis of affinity-purified antigen.** From Western blot analysis of total BMC lysates, it was difficult to define unequivocally any particular protein bands as specifically immunoreactive with the 175 MoAb. However, fractionation of the lysate by 175 MoAb affinity chromatography showed a closely associated protein doublet band within the range of 28 to 30 kD that reacted with the 175 MoAb on subsequent immunoblot analysis run under either reducing or nonreducing conditions (Fig 1). The immunoreactivity of the upper 30-kD band was considerably reduced, whereas that of the lower 28-kD band was enhanced by digestion of the sample before separation with the endoglycosidase PNGase-F (Fig 2). The 175 antigen will therefore be referred to as a 28-kD glycoprotein.

![Fig 1](image-url) Western blot analysis of the bound protein fraction eluted from a 175 MoAb-Sepharose column. The membrane was probed with (a) an IgM fraction of VPM12 anti-BDV control MoAb, and (b) an IgM fraction of 175 MoAb. Bound MoAb was detected by subsequent incubation with sheep anti-IgM antibodies conjugated with horseradish peroxidase and visualized by addition of ECL reagent and exposure to x-ray film. Two closely associated bands of approximately equal intensity were identified on the original gel. The 17-kD band (the α-chain of hemoglobin) was bound nonspecifically to the 175 MoAb.
In some analyses, a 17-kDa band was also observed (Fig 1). A 20–amino acid N-terminal sequence analysis showed homology to the α-chain of hemoglobin. This bound nonspecifically to 175 MoAb and a selection of other IgM and IgG control murine MoAbs (Fig 3).

N-terminal sequence analysis. Increased resolution of the protein doublet band was achieved by running gels of greater length and at a higher acrylamide concentration (12%). Following analysis, both the 28- and 30-kDa bands were found to have the same N-terminal amino acid sequence over 20 residues. This sequence was compared with those filed with the NBFR FIR1-3 Protein Sequence Library. The search showed complete homology over 20 amino acids at the N-terminus between the 28-kD antigen and that of a serine protease of bovine origin (Table 1).

Cell-surface expression of the 28-kD protein. To demonstrate that the 28-kD protein isolated from BMC lysates corresponded to the cell-surface antigen immunoreactive with the 175 MoAb in flow-cytometric analysis of viable BMC, radiolabeled BMC surface proteins were analyzed after immunoprecipitation (Fig 3). In addition to the expected 28-kD component, a less intense band of approximately 55 kDa was also detected. The 17-kD hemoglobin chain also bound nonspecifically to the 175 MoAb and to a lesser extent the control IgM MoAb (Fig 3).

Protease activity of the 28-kD protein. The affinity-purified 28-kD protein was associated with protease activity as shown in azocasein substrate gels (Fig 4). Furthermore, activity was inhibited only by the serine protease inhibitor PMSF and not the metallo, thiol, or acid protease-specific inhibitors. The 28-kD antigen proteolytic activity was also inhibited by inclusion of the 175 MoAb in the substrate gel (Fig 5). Inhibition of protease activity in the 28- to 30-kD range in total BMC lysates was only partially blocked when compared with the affinity-purified material. The activities of a selection of other serine proteases in the substrate gels were not inhibited by the 175 MoAb.

### Table 1. N-Terminal Sequence Identities Between the 175, 28-kD Protein and Other Serine Proteases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>N-Terminal Sequence</th>
<th>Identity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>175, 28-kD</td>
<td>Ovine bone marrow</td>
<td>IVGRKARPOELPFLASIGN</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>28-kD serine protease</td>
<td>Bovine pituitary</td>
<td>IVGRKARPOELPFLASIGN</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>Azurocidin (CAP37, hHBP)</td>
<td>Human neutrophil</td>
<td>IVGRKARPOELPFLASIGN</td>
<td>85</td>
<td>13, 14</td>
</tr>
<tr>
<td>Porcine heparin-binding protein</td>
<td>Porcine neutrophil</td>
<td>IVGRRARQPDNEPFLASIQK</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>Leukocyte elastase</td>
<td>Human neutrophil</td>
<td>IVGRRARPHAWPMVMQL</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Proteinase-3 (MB, WA)</td>
<td>Human neutrophil</td>
<td>IGGHAQNSRHPYMASL</td>
<td>58</td>
<td>13, 16</td>
</tr>
</tbody>
</table>

Alternative names in parentheses.
Abbreviations: CAP37, cationic antimicrobial protein; hHBP, human heparin-binding protein; MB, myeloblastin; WA, Wegener’s autoantigen.
The 175 antigen shared an identical N-terminal 20–amino acid sequence to a serine protease that coprecipitated along with basic fibroblast growth factor from bovine pituitary cells immunoreactive with an antibody to basic fibroblast growth factor. The 175 MoAb reacts with a population of cells that line the sinuses of ovine pituitary (unpublished data), but initial attempts to isolate a complex of 175 antigen with lower–molecular weight proteins on heparin-sepharose, as described in the bovine, have been unsuccessful.

Serine proteases are ubiquitous enzymes with multiple activities on cell and tissue development and function, and cell-surface proteases may play a role in cell differentiation, migration, and control of inflammation. The 175 protease has a different cellular distribution to other reported proteases on hematopoietic cells. High levels of expression are seen on bone marrow or tissue monocytes and macrophages, neutrophils, eosinophils, stromal and fibroblast cells, and bone marrow erythroid series cells. However, expression of 175 antigen is absent (or below detectable levels) on mast cells, basophils, and the majority of lymphocytes. Expression of 175 antigen increases on hematopoietic progenitor cells as they develop from precursors of CFC (undetectable level) via more lineage-restricted CFC to differentiated BMC. The 175 antibody has therefore proven useful in a cocktail of antibodies used to deplete more mature BMC, leaving a highly enriched population of early clonogenic CFC and their precursors.

The significance of the distribution of the 175 antigen and the initiation of functional studies must await the cloning of the cDNA, which is currently underway. In addition, it will be of interest to identify whether there is an equivalent human antigen.

**REFERENCES**

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D Deane, L Inglis and D Haig