We studied endosomal proteolysis of the surface fibrillar M5 protein from viable Streptococcus pyogenes as an essential step involved in major histocompatibility complex class II-restricted antigen processing of two immunodominant CD4+ T-cell epitopes (17–31/Eα and 308–319/Aβ). Intracellular proteolysis of viable streptococci for presentation of 17–31, bound by serine proteinase cleavage sites, was mediated by serine proteinases, whereas processing of soluble recombinant M5 protein required in addition cysteine proteinases. Furthermore, processing of 17–31 was resistant to ammonium chloride and thus was not dependent on endosome acidification. Cysteine and serine proteinase cleavage sites were located adjacent to 308–319, and its processing was dependent on serine, cysteine, and aspartic proteinases, as well as on endosomal acidification. The data suggest that antigen processing of two major T-cell epitopes on streptococcal M5 protein occurred in different endosomal compartments by different classes of intracellular proteinases.

Exogenous antigens have to be subjected to intracellular enzymatic processing by professional antigen-presenting cells for major histocompatibility class II (MHC-II)-dependent presentation of peptide fragments to CD4+ T-cells (1). Peptides released in late endosomes are transported to specialized MHC-II binding compartments for recognition by MHC-II molecules (2, 3). A chaperone molecule (the invariant (Ii) chain) associated with MHC-II molecules targets the nonameric αβ-Ii complex via endoplasmic reticulum and trans-Golgi network to endosomes. Ii chain is then sequentially cleaved by aspartic and cysteine proteinases, and HLA-DM (H2-M in mouse) facilitates the exchange of the class II-associated invariant chain peptide for processed antigenic peptides (4). Recent evidence suggests that antigen processing of certain proteins (influenza hemagglutinin and myelin basic protein) can also occur in early endosomal compartments with peptides being loaded on MHC-II molecules recycled from the plasma membrane (5, 6) to specialized class II vesicles.

Intracellular proteolytic enzymes have been shown to be intrinsically associated with antigen processing (7). Indeed, at least two out of four classes of intracellular endopeptidases, namely aspartic and cysteine proteinases, have been shown to be directly involved in endosomal proteolysis of model antigens as well as in enzymatic processing of Ii chain (2, 7–9). Thus, cathepsin S has been reported to be primarily engaged in degradation of Ii chain, thus facilitating the downstream peptide loading on MHC-II molecules (10). However, other classes of intracellular enzymes, i.e. metalloproteinases, may contribute to the overall efficiency of antigen processing (11). It has also been shown that the proteolytic activity of endosomal enzymes is regulated by the pH gradient within the endosomal pathway (7, 12, 13), suggesting that different classes of proteinases may be implicated in distinct MHC-II antigen processing compartments.

We have previously shown that two CD4+–dependent T-cell epitopes (17–31/Eα and 308–319/Aβ) located on the surface fibrillar M5 protein, the main virulence factor and protective antigen of Streptococcus pyogenes, were efficiently processed from viable streptococci for MHC-II-restricted presentation to specific CD4+ T-cell clones and T-cell hybridomas (14–17). In this report we present evidence that processing of 17–31 was mediated by serine proteinases, whereas 308–319 required serine, cysteine, and aspartic proteinases, suggesting that two epitopes on the same protein engage largely different endosomal compartments for MHC-II-dependent antigen processing.

**Experimental Procedures**

**Cells and Chemicals**—The murine macrophage cell line J774A.1 (H-2d, ATCC TIB 67) was used as antigen-presenting cells. T-cell hybridomas (HX17 and HY2) obtained by polyethylene glycol fusion of two M5 protein-specific T-cell clones (X17 and Y2) with BW5147 (TCRαβ) cells (kindly provided by Dr. P. Marrack, Denver) were specific for epitopes 17–31/Eα and 308–319/Aβ of group A streptococcal type 5 M protein, respectively, as reported previously (17). Antigen-presenting cells and T-cell hybridomas were grown in RPMI 1640 medium containing 3.0 mm L-glutamine, 0.05 mm 2-mercaptoethanol, and 10% fetal bovine serum (v/v). S. pyogenes (strain Manfredo) was grown overnight in RPMI 1640 with 10% fetal bovine serum, washed once in phosphate-buffered saline and the concentration was adjusted spectrophotometrically to 3 × 10⁵ colony-forming units/ml (A₆₀₀ = 0.6). Culture media, chemicals, and metabolic inhibitors (Table I) were from Sigma Chemical Co. (Dorset, UK).

**Soluble Recombinant M5 Protein (rM5) and Synthetic Peptides**—The recombinant M5 protein (rM5) from type 5 S. pyogenes strain Manfredo was cloned and expressed in Escherichia coli LE392 as described previously (14, 18). Synthetic peptides covering two T-cell epitopes on the M5 protein of S. pyogenes were purchased from the University of Newcastle upon Tyne, Facility for Molecular Biology: (i) 15–33 peptide contained epitope 17–31/Eα, and (ii) 300–319 peptide covered epitope 308–319/Aβ (14).

**Antigen Processing Assay**—Following adherence (6 × 10⁵well) (48-well plates, Bibby Sterlin Ltd., Staffordshire, UK) for 1 h, J774A.1 macrophages were treated with inhibitors for 30 min, unless stated otherwise. Viable streptococci (3 × 10⁵well), rM5 (1.0 µg/ml), or syn-
thetic peptides (4.0 μg/ml) were added, and plates were incubated at 37 °C in a humidified CO₂ incubator for 1 h, after which nonphagocytosed bacteria were killed with gentamycin (50 μg/ml), and the plates were incubated for an additional 3 h. The macrophages were then fixed with 1.0% paraformaldehyde for 10 min, washed with phosphate-buffered saline, and T-cell hybridoma cells were added (3 × 10⁴/well) for 24 h. The culture supernatants were removed and stored at −20 °C for subsequent interleukin-2 assay. To ensure that the inhibition observed resulted from the specific, rather than nonspecific, effect of inhibitors, the viability of J774A.1 cells before fixation was confirmed in all experiments. All experiments were performed at least three times, and the data for a representative experiment are shown.

The response of T-cell hybridomas was measured as proliferation of CTLL-2 cells (10⁴/well) in the presence of T-cell hybridoma culture supernatants in flat-bottomed 96-well microtiter plates (Becton Dickinson Labware, New Jersey). Each supernatant was tested at a 1:2 dilution in duplicate for 24 h at 37 °C in a humidified CO₂ incubator, followed by pulse-labeling with 0.4 mCi of [3H]thymidine (TRA310, specific activity 2.0 Ci/mmol; Amersham International plc, Buckinghamshire, UK) for 18 h. Cells were harvested on glass fiber membranes, and radioactivity was quantitated using a direct beta counter (Matrix 9600, Packard Instrument Company, Meridan, CT).

RESULTS

Role of Endosome Acidification in Antigen Processing—For antigen processing to take place, both the enzyme and the appropriate pH should be present within one endosomal compartment of the antigen-presenting cells to ensure efficient proteolysis. It has been shown that although intracellular proteinases are present in all endocytic compartments, to attain full enzymatic activity inactive proenzymes require exoproteolytic maturation controlled by the pH gradient within the endosomal pathway (7, 19). Hence, endosomal acidification plays a pivotal role in antigen processing and presentation.

To study the importance of endosomal acidification for processing of the streptococcal M5 protein for presentation of two immunodominant T-cell epitopes (17–31/Ed and 308–319/Ad) to...
specific T-cell hybridomas, we used two specific metabolic inhibitors. Monensin is a carboxylic (cationic) ionophore (20) that intercalates into membranes and mediates exchange of protons for potassium ions, thus effectively raising endosomal pH (12). Ammonium chloride is a weak base that promotes alkalinization of the endosome content (12).

Ammonium chloride had no apparent effect on presentation of 17–31 (Fig. 1A). In contrast, this inhibitor blocked processing of 308–319 from viable bacteria or rM5 protein, and reduced presentation of the relevant synthetic peptide. Monensin exhibited a profound inhibitory effect on presentation of 308–319 from both viable streptococci and soluble rM5, whereas only partial reduction of 17–31 presentation was recorded in the same experiments (Fig. 1B). This data suggested that endosome acidification is critical for processing of 308–319, whereas processing of 17–31 was less dependent on endosomal pH.

Endosomal Proteolysis by Cysteine Proteinases—There is evidence to suggest that cysteine proteinases mediate antigen processing of some model antigens, such as sperm whale myoglobin (8, 21), pigeon cytochrome c (22), tetanus toxin (23), synthetic oligopeptides (24), and hen egg lysozyme (11) but not ovalbumin (9, 25). To study the role of this class of proteinases in antigen processing, we used (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane, a membrane permeable inhibitor that reacts with a cysteine residue at the enzyme active center selectively inactivating cysteine proteinases (cathepsins L, S, and B) (13, 26). This inhibitor blocked processing of 308–319 from viable streptococci and rM5 and processing of 17–31 from soluble rM5 but not from bacteria (Fig. 2A). Similarly, p-hydroxymercuribenzoic acid, an irreversible cysteine proteinase inhibitor, blocked processing of 308–319 and caused only a marginal inhibition in presentation of 17–31 from rM5 (Fig. 2B). Thus, processing of viable streptococci for 17–31 presentation was not dependent on cysteine proteinase activity, whereas this class of intracellular enzymes was critical for processing of 308–319 from bacteria and rM5.

Involvement of Other Classes of Proteinases in Antigen Processing—Data in Fig. 3 show the effect of other inhibitors on antigen processing. Na-p-tosyl-l-lysine chloromethyl ketone (TLCK) and N-tosyl-l-phenylalanine chloromethyl ketone (TPCK) were used to inactivate trypsin-like and chymotrypsin-like serine proteinases, respectively (8), and phenylmethylsulfonyl fluoride to block most serine proteinases. A peptide aldehyde leupeptin, which is known to react with serine/cysteine residues at enzyme active centers forming hemiacetal or hemithioacetal groups, was employed to inactivate both serine and cysteine proteinases (13) (Fig. 3, A-D). We observed that TLCK and TPCK exerted a consistent blocking effect on processing of both epitopes (Fig. 3, A and C). Phenylmethylsulfonyl fluoride inhibited 308–319 and marginally suppressed 17–31 presentation (Fig. 3B). The effect of leupeptin on antigen processing was largely similar to that of (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane in that leupeptin blocked processing of 17–31 from soluble rM5 and 308–319 from both viable bacteria and rM5 (Fig. 3D). Further data show that 1,10-phenanthroline, which blocks metalloproteinases, did not interfere with antigen processing of both epitopes (Fig. 4B). No cleavage sites recognized by aspartic proteinases were present near both.

**Fig. 3.** Effect of inhibitors of serine proteinases on presentation of two M5 protein T-cell epitopes. Presentation of two M5 protein-specific T-cell epitopes (17–31 and 308–319) from viable streptococci (squares), rM5 (diamonds), or synthetic peptides (circles) to specific T-cell hybridomas, HX17 (closed symbols) and HY2 (open symbols). Different concentrations of TLCK (A), phenylmethylsulfonyl fluoride (PMSF) (B), TPCK (C), and leupeptin (D) are shown as labels of the x axis. Other details are as in the legend to Fig. 1.
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FIG. 4. Effect of inhibitors of other intracellular proteinases on presentation of two M5 protein T-cell epitopes. Presentation of two M5 protein-specific T-cell epitopes (17–31 and 308–319) from viable streptococci (squares), rM5 (diamonds), or synthetic peptides (circles) to specific T-cell hybridomas, HX17 (closed symbols) and HY2 (open symbols). Different concentrations of pepstatin (A) or 1,10-phenanthroline (B) are shown as labels of the x axis. Other details are as in the legend to Fig. 1.

DISCUSSION

We have previously shown that like globular proteins, the surface fibrillar M5 protein of group A streptococci needs intracellular antigen processing for efficient presentation of two immunodominant CD4+ T-cell epitopes studied. The underlined sequences correspond to the synthetic peptides used, and sequences marked in bold bound the epitope structure. Arrows point to enzyme cleavage sites as follows: chymtr., chymotrypsin-like serine proteinases that cleave between F–X, W–X, Y–X (13, 27); tr., trypsin-like serine proteinases that cleave between K–X, R–X (13, 27); cath.B, cathepsin B, a cysteine proteinase that cleaves between K–K, K–R, R–R, or after F–R (13, 30); and cath.S, cathepsin S, a cysteine proteinase that cleaves between K–L, K–V (31). No aspartic proteinase cleavage sites (cleave between F–F, F–Y, and L–F (13)) were found in the vicinity of either epitope.

Evidence was obtained that engagement of a particular class of proteolytic enzymes in antigen processing of two epitopes on the streptococcal M5 protein was dependent on both the amino acids flanking the epitope and the form of antigen delivery (viable bacteria or soluble rM5). Indeed, our data suggested that processing of 17–31 from bacteria was mediated by serine proteinases and was not dependent on endosome acidification, consistent with the presence of serine proteinase cleavage sites located adjacent to this epitope (Fig. 5) and with the neutral pH optimum for serine proteinase activity (13, 27). Interestingly, soluble rM5 protein required both serine and cysteine proteinases to facilitate processing of 17–31 for presentation to specific T-cells. In the absence of cysteine proteinase cleavage sites in the vicinity of 17–31, the effect of cysteine proteinases could be restricted to Ii chain proteolysis, which is essential for antigen presentation with newly synthesized MHC-II molecules of the classical antigen processing pathway (1, 2). Collectively, the data imply that antigen processing of viable streptococci and soluble rM5 protein for 17–31 presentation occurred largely in different endosomal compartments, early and late endosomes, respectively. Targeting of rM5 to distinct MHC-II processing compartments in this case would be expected if the polypeptide chain of this recombinant protein needed unfolding prior to proteolytic cleavage as has been described for native but not denatured sperm whale myoglobin (21). In contrast, the N-terminal 17–31 epitope of the M5 protein expressed on the bacterial cell surface could have been cleaved by plasma membrane-associated endopeptidases as described for processing of bovine serum albumin by the A20 lymphoblastoid cell line (28) and routed to early endosomes. It is not clear if presentation of 17–31 after processing of whole bacteria occurred in the context of a large antigen fragment or whether it was trimmed by serine proteinases after binding to MHC-II molecules thus protecting the trypsin-like serine proteinase cleavage site within the epitope (Fig. 5).

Processing of 308–319 from both rM5 and bacteria was mediated by serine, cysteine, and aspartic proteinases and was found to be dependent on endosome acidification. The data suggests engagement of late endosomes/lysosomes that provide the necessary acidic environment for acquisition of full enzymatic activity of cysteine and aspartic proteinases (7, 19). Dependence of 308–319 processing on aspartic proteinases is not consistent with the absence of aspartic proteinase cleavage sites in the vicinity of this epitope, again suggesting that aspartic proteinases exerted an indirect effect on antigen proc-
cessing via the previously described inhibition of sequential proteolysis of Ii chain from (αβ)IIα complexes (2, 4, 29).

Data presented herein indicate that two immunodominant T-cell epitopes on the streptococcal M5 protein engage different endosomal compartments and required different classes of intracellular proteolytic enzymes for antigen processing. Knowledge of the mechanisms of enzymatic cleavage of protective antigens during antigen processing of T-cell epitopes from viable bacteria has important implications for live vaccine delivery systems.

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Different Endosomal Proteolysis Requirements for Antigen Processing of Two T-cell Epitopes of the M5 Protein from Viable Streptococcus pyogenes
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