Identification and localization of an iron-regulated 35 kDa protein of *Pasteurella haemolytica* serotype A2

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Iodination of intact *Pasteurella haemolytica* serotype A2 cells labelled a sub-set of total cellular proteins. Comparison of the autoradiographic patterns obtained from iodinated cells grown on complete medium and on iron-depleted medium showed that expression of three proteins, of 100, 70 and 35 kDa, respectively, was increased by growth under iron-depleted conditions. Of these proteins, that of 35 kDa had not been reported previously. Like the 100 and 70 kDa proteins, the 35 kDa protein was expressed in natural infections, since it was recognized by antiserum from sheep that had recovered from an experimental infection with *P. haemolytica* A2. The 35 kDa protein was partially purified by reverse-phase HPLC and was found to be antigenic in both sheep and mice. A monoclonal antibody that was specific for the 35 kDa protein was used to identify the cellular location of the protein by immunoblotting of cell fractions enriched for particular cellular components. This demonstrated that the 35 kDa protein was located mainly in the periplasm.

Introduction

Pasteurella haemolytica comprises sixteen distinct serological types, which can be identified by an indirect haemagglutination (IHA) test (Biberstein *et al.*, 1960; Fodor *et al.*, 1988; Fraser *et al.*, 1982). In Britain, the A2 serotype is most prevalent, being responsible for pneumonic disease in all ages of sheep, and septicaemia with high mortality in lambs.

Sheep which have recovered from experimental infection with *P. haemolytica* A2 are immune to subsequent challenge (Donachie *et al.*, 1986). However, experimental vaccination with killed A2 cells which have been grown *in vitro* in a complete culture medium fails to elicit the same degree of resistance (Gilmour *et al.*, 1983), despite producing a humoral response (Jones *et al.*, 1989). One possible reason for this is that important immunogenic components are expressed by cells *in vivo*, but not by cells cultured on a complete medium *in vitro*. The iron-regulated proteins (IRPs) may represent one class of components of this nature. These proteins are strongly expressed under conditions where iron availability is restricted, as is the case in the animal host *in vivo* or on iron-depleted medium *in vitro* (Neilands, 1982; Griffiths, 1987; Payne, 1988). Dona-

chie & Gilmour (1988) have reported two IRPs of 70 and 100 kDa, located in the outer membrane of *P. haemolytica* A2 cells isolated directly from the pleural fluid of infected sheep. These proteins were expressed at a low level in A2 cells grown *in vitro* on iron-replete medium, but could be induced to higher levels of expression when the cells were grown instead on a medium which had been depleted of iron with an iron-chelating agent. Serum from convalescent sheep contained antibodies to both of these IRPs, indicating that they were expressed in natural infections and were antigenic. Recently, Deneer & Potter (1989) described the presence of a third outer-membrane IRP of 77 kDa in *P. haemolytica* serotype A1. This was in addition to IRPs of 71 and 100 kDa, which presumably were the same as those previously reported by Donachie & Gilmour (1988) for serotype A2.

Here we describe a further iron-regulated protein, 35 kDa in size, of *P. haemolytica* serotype A2. This protein is located mainly in the periplasmic space, is naturally present *in vivo* and can be induced to a high level of expression *in vitro*.

Methods

Bacterial cells. *P. haemolytica* A2 (strain A713) cells were grown in nutrient broth (Gibco) at 37 °C with shaking. Medium depleted of free iron was obtained by the addition of 2,2'-dipyridyl (Sigma) at a final

Abbreviations: IRP, iron-regulated protein; SPF, specific-pathogen-free.

concentration of 150 mM. Cells were collected by centrifugation at 3000 g for 15 min and then washed by two cycles of centrifugation and resuspension in phosphate-buffered saline (PBS) (pH 7.5) to remove culture medium. A2 cells grown *in vivo* were recovered directly from the pleural fluid of specific-pathogen-free (SPF) lambs suffering from pneumonic pasteurellosis, as described by Donachie & Gilmour (1988).

Cell surface iodination. *P. haemolytica* cells were grown with shaking at 37 °C to an OD₆₀₀ of 0.5, collected by centrifugation at 3000 g for 15 min, washed once and then finally resuspended in an equal volume of phosphate-buffered saline (PBS). A 0.2 ml aliquot of this suspension was removed and cooled on ice, and 200 µCi (7.4 MBq) of carrier-free ¹²⁵I (Na salt) was added together with two iodobeads (Pierce). The mixture was incubated on ice for 10 min with frequent gentle mixing and the reaction terminated by removing the cell suspension to a fresh tube. Unincorporated iodine was then removed by several cycles of centrifugation and resuspension in PBS.

Gel electrophoresis. Samples were dissolved in sample buffer [2% (w/v) sodium dodecyl sulphate (SDS), 5% (w/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.002% bromophenol brilliant blue and 125 mM-Tris/HCl, pH 6.8], heated in a boiling water bath for 5 min and then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (w/v) gel (Laemmli, 1970). Proteins were detected by Coomassie-blue staining. Subsequently, the gel was dried on a heated slab-gel drier (Bio-Rad) and autoradiography carried out at -70 °C.

Immunoblotting. SDS-PAGE gels were electroblotted onto nitrocellulose (Towbin *et al.*, 1979). Filters were blocked with 50% (v/v) horse serum in PBS for 2 h at room temperature with gentle agitation. For detection, filters were incubated for 1 h at room temperature with a monoclonal antibody, or an antiserum appropriately diluted in phosphate-buffered saline (PBS) containing 10% (v/v) horse serum and 0.5% Tween 20, and were then given 5 × 10 min washes in PBS containing 0.5% Tween 20. Subsequently, the filters were incubated with horseradish-peroxidase-conjugated anti-mouse or anti-sheep Ig (Scottish Antibody Production Unit) and then again extensively washed as described above. Colour development was carried out at room temperature in 100 mM-Tris-buffered saline (TBS) (pH 7.6), containing diaminobenzidine at 0.5 mg ml⁻¹ and 2 µl ml⁻¹ of 30% (v/v) H₂O₂. The reaction was terminated by washing the filters in tap water.

High-pressure liquid chromatography. Protein separations were achieved using a PLRP-100A (5 µm) 250 mm × 4.6 mm reversed-phase chromatography column (Polymer Laboratories). Bacterial samples were prepared by mixing 198 µl of *P. haemolytica* cell pellet suspended 1:1 in PBS with 396 µl 8 M-guanidine hydrochloride and 6 µl 2-mercaptoethanol in an Eppendorf tube. The mixture was vortexed for 1 min and then centrifuged at 11 000 g for 10 min. Clear supernatant (400 µl) was applied to the column and the proteins eluted using a gradient containing two components: A, an aqueous solution of 0.1% (w/v) trifluoroacetic acid (TFA) and B, the eluant, acetonitrile containing 0.1% TFA. The column was first equilibrated with 99% A:1% B. Proteins were then eluted selectively by a linear gradient in which the eluant (B) was held at 1% for 5 min, increased to 5% between 5 and 10 min, and subsequently increased linearly to 95% by 170 min. The flow rate was maintained at 1 ml min⁻¹. Fractions were collected at 1 min intervals and the A₂₈₀ of the eluate recorded. The fractions were neutralized with 14 µl 1 M-NaOH, and 100 µl aliquots were dried in a stream of dry air and prepared for analysis by SDS-PAGE.

Production of an anti-35 kDa monoclonal antibody. On days 0 and 14 a Balb/c mouse was inoculated intraperitoneally with 5 × 10⁸ *P. haemolytica* A2 (A713) cells in a Bayol/Alhydrogel adjuvant (Wells *et al.*, 1979). On day 49, 50 µg of semi-purified 35 kDa antigen was inoculated intraperitoneally in adjuvant. A positive response to immunization was demonstrated by reaction of the mouse serum at

1/100 dilution with the 35 kDa antigen on an immunoblot. On day 98 immunity was boosted by intravenous inoculation of 50 µg semi-purified 35 kDa antigen in PBS, and on day 101 the spleen was removed. Cells from the spleen were fused with the plasmacytoma line NS-0 by the method of Oi & Herzenberg (1980). Supernatant fluids from the resulting hybridoma cell lines were screened by a solid-phase ELISA assay using *P. haemolytica* cells or semi-purified 35 kDa antigen. Positive hybridoma fluids were tested further for anti-35 kDa activity by immunoblotting. One line cloned by limiting dilution (Ph12/16) continuously secreted antibody directed against the 35 kDa antigen.

Fractionation of *P. haemolytica* cells. The methods used were based on those described by Witholt *et al.* (1976) and Middeldorp & Witholt (1981). Details of this procedure are given below. Eighty milligrams of freeze-dried cells prepared from PBS-washed *P. haemolytica* A2 (A713) were suspended in 1 ml 200 mM-Tris/HCl buffer (pH 8.0) and incubated at 22 °C for 10 min. One ml TESL buffer [200 mM-Tris/HCl, 1 M-sucrose, 0.5 mM-EDTA and 120 µg lysozyme (Sigma) ml⁻¹] was then added, and the mixture was incubated for 5 min and then diluted 1:1 with distilled water before incubation at 22 °C for a further 30 min with vigorous shaking. DNAase (Sigma) was next added to give 50 µg ml⁻¹ and the suspension centrifuged at 15 000 g for 60 min to pellet the spheroplasts, which were washed once in TESL. Aprotinin (Sigma), was added at 1.5 IU ml⁻¹ to the supernatant (the periplasmic contents), which was then stored at -20 °C. The pelleted spheroplasts were resuspended in 5 ml osmotic stabilizing buffer (OSB), 10 mM-Tris/HCl (pH 8.0) containing Aprotinin (1.5 IU ml⁻¹), DNAase (50 µg ml⁻¹) and RNAase (Sigma) (50 µg ml⁻¹) and sonicated by 5 × 30 s pulses at full power using an MSE sonicator with a 1 cm diameter probe. The samples were cooled in an ice bath between sonication pulses. Disrupted spheroplasts were centrifuged at 5000 g for 10 min to pellet unlysed cells, which were stored at -20 °C. The supernatant (cell envelopes plus cytoplasmic contents) was centrifuged at 30 000 g for 45 min to pellet envelopes, which were washed once in 10 mM-Tris/HCl (pH 7.6) and resuspended in 1 ml of the same buffer. The first supernatant (the cytoplasmic contents) was stored at -20 °C. The envelope pellet was incubated at room temperature in 10 mM-Tris/HCl (pH 7.6) containing 2% (w/v) Sarkosyl (sodium laurylsarcosinate, Sigma), for 30 min and then centrifuged at 190 000 g for 90 min to pellet outer-membrane components, which were washed once in buffer. The supernatant and pellet were stored at -20 °C. The periplasmic contents and cytoplasmic contents were also subsequently centrifuged at 190 000 g for 90 min and the pellet and supernatant from each stored at -20 °C.

Antisera from convalescent sheep. Antisera were obtained from SPF lambs that had recovered from experimental A2 infection (Donachie *et al.*, 1986).

Immune serum from sheep. Two SPF lambs were immunized with semi-purified 35 kDa protein prepared by HPLC separation. Each lamb was immunized with a total of 550 µg protein absorbed on to 9% (w/v) alhydrogel (Superphos, Denmark) in Tris/HCl (pH 7.4). Each animal received the antigen subcutaneously in two doses, the first of 420 µg on day 0 and the second, administered 28 days later, of 130 µg. Serum samples were taken before inoculation on days 0 and 28 and then on days 42 and 57.

Results

Identification of iron-regulated protein of 35 kDa

The rate of growth and final yield of cells was very similar in cultures of *P. haemolytica* grown on iron-replete (+Fe) medium and on iron-deficient (-Fe)

Fig. 1. SDS-PAGE and autoradiography of *P. haemolytica* A2 whole-cell lysates. Proteins from surface-iodinated cells were separated by SDS-PAGE and stained with Coomassie blue: lane 1, cells grown in iron depleted medium ($-Fe$) medium; lane 2, cells grown in iron replete ($+Fe$) medium. The resulting autoradiograph: lane 3, cells grown in $-Fe$ medium; lane 4, cells grown in $+Fe$ medium. The positions of the 100, 70 and 35 kDa proteins are indicated.

medium. Cells grown on $+Fe$ medium and $-Fe$ medium were iodinated and lysates analysed by SDS-PAGE and stained with Coomassie blue. A complex banding pattern was obtained for each lysate (Fig. 1, lanes 1 and 2). However, the patterns clearly differed in that a band at 35 kDa was much more intense in the lysate of cells grown on the $-Fe$ medium. Subsequent autoradiography of the gel indicated that substantial incorporation of radioactivity had occurred, resulting in the labelling of approximately 20 proteins in each of the two lysates. However, once again, differences in the banding patterns were observed, with bands of 100 kDa, 70 kDa and 35 kDa being much more evident in the track derived from the cells grown on the $-Fe$ medium (Fig. 1, lanes 3 and 4). The iodination procedure predominantly labels proteins that are located peripherally in whole cells. This experiment indicated, therefore, that three such proteins were being expressed much more

Fig. 2. Fractionation of *P. haemolytica* whole-cell lysates by reversed-phase HPLC: elution profiles of *P. haemolytica* A2 cells grown in $-Fe$ medium (trace 1) or in $+Fe$ medium (trace 2). The A_{280} of the eluate was monitored and fractions corresponding to the 35 kDa peak were collected. The position of the 35 kDa peak is indicated.

strongly under conditions of iron depletion than under conditions of iron sufficiency. The 100 kDa and 70 kDa IRPs have previously been reported (Donachie & Gilmour, 1988); the 35 kDa protein is described here.

Purification of the 35 kDa protein

Fig. 2 shows the elution profiles of *P. haemolytica* whole-cell lysates separated by reversed-phase HPLC. Cells grown in $-Fe$ medium (trace 1) and in $+Fe$ medium (trace 2) had comparable elution profiles but could be distinguished by a single prominent peak that was expressed in cells grown in $-Fe$ medium. Samples of eluate fractions encompassing this peak were separated by SDS-PAGE and stained with Coomassie blue. As is evident from Fig. 3, the fractions contained mainly a protein of 35 kDa, although some other minor components were also present. Fractions 3 and 4, containing the bulk of the 35 kDa protein, were pooled for further analysis. Corresponding fractions from A2 cells grown in $+Fe$ medium contained very much reduced amounts of this protein (Fig. 3).

Reactivity of convalescent sheep antiserum with the 35 kDa protein

Convalescent antiserum contained antibodies directed against a 35 kDa antigen. In immunoblotting analysis of whole *P. haemolytica* A2 cell preparations, these antibodies reacted with an antigen of 35 kDa present in cells grown in $-Fe$ medium (Fig. 4, lane 1), but only weakly with the same antigen from cells grown in $+Fe$ medium (Fig. 4, lane 2). The observation that the 35 kDa antigen recognized by convalescent antiserum was

Fig. 3. Analysis of HPLC fractions (see Fig. 2) by SDS-PAGE and Coomassie blue staining. (a) Fractions 1 to 7 from cells grown in -Fe medium. (b) Corresponding fractions from cells grown in +Fe medium. The position of the 35 kDa protein is indicated (arrow).

'iron-regulated' implies that it is the same 'iron-regulated' protein that was identified in Figs 1 and 3.

Reactivity of an anti-35 kDa monoclonal antibody

In immunoblotting analysis the monoclonal antibody Ph12/16 reacted strongly with a single 35 kDa antigen in lysates of whole cell preparations of *P. haemolytica* A2 grown in -Fe medium (Fig. 5, lane 2) or cultured *in vivo* (Fig. 5, lane 4). In contrast, reaction with this antigen was greatly diminished in lysates of cells grown in +Fe medium (Fig. 5, lane 1). Apart from some slight background reaction, there were no cross-reactions between the anti-35 kDa monoclonal antibody and other antigens of *P. haemolytica*. The anti-35 kDa monoclonal antibody also reacted strongly with the major component of the partially-purified 35 kDa protein (Fig. 5, lane 3). This confirmed that the dominant specificity of the antibody was indeed for the 35 kDa protein.

Development of an immune response to the partially purified 35 kDa protein in SPF lambs

The development of an immune response to vaccination with the 35 kDa protein was monitored by immunoblotting using the pooled serum from two lambs. The antigens used in the blots were (i) whole cells grown in -Fe medium (Fig. 6, lanes labelled A) and (ii) the partially purified 35 kDa protein used for immunization of the lambs (Fig. 6, lanes labelled B). The pre-immune serum (day 0) showed no reaction with the 35 kDa antigen, although some cross-reaction with other proteins in the whole cell lysate was observed. On day 28 a clear response to the primary inoculating dose of 35 kDa protein was visible in whole-cell and partially purified preparations. This response was even stronger on days 42 and 57, although at this stage residual contamination of the immunizing preparation with other cell components resulted in strong reactions with other antigens.

Fig. 4. Immunoblotting analysis of the reactions of convalescent antisera with *P. haemolytica* whole-cell lysates. Lane 1, A2 cells grown in $-Fe$ medium; lane 2, A2 cells grown in $+Fe$ medium.

Localization of the 35 kDa protein

Subcellular fractions of *P. haemolytica* A2 cells grown in $-Fe$ medium were prepared. They were then probed with the anti-35 kDa monoclonal antibody Ph12/16 in an immunoblotting experiment. Fig. 7 shows that the antibody reacted strongly with lysates of whole cells (lane 1), the periplasmic contents (lanes 4 and 5) and, to a much lesser extent, the cytoplasmic contents (lanes 2 and 3). This indicates that the 35 kDa antigen is located principally within the periplasmic space. The 35 kDa antigen was only a minor component of the membrane fractions (Fig. 7, lanes 6, 7 and 8).

Discussion

Two previous studies have identified 'iron-regulated' proteins in the A2 serotype of *P. haemolytica*. Donachie & Gilmour (1988) described two iron-regulated outer

Fig. 5. Specificity of the anti-35 kDa monoclonal antibody Ph12/16 against the 35 kDa IRP of *P. haemolytica*, as determined by immunoblotting. Lane 1, whole-cell lysate, cells grown in $+Fe$ medium; lane 2, whole-cell lysate, cells grown in $-Fe$ medium; lane 3, 35 kDa protein partially purified by HPLC; lane 4, whole-cell lysate, cells isolated from the pleural fluid of infected lambs.

membrane proteins of 70 kDa and 100 kDa and Deneer & Potter (1989) described an additional one of 77 kDa. Although no function has been ascribed to these proteins they are expressed *in vivo* in natural infections and their synthesis can be induced *in vitro* in conditions of iron deprivation.

This study provides evidence for the presence of a further 35 kDa 'iron-regulated' protein which is expressed in the periplasmic space of *P. haemolytica* serotype A2. The 35 kDa IRP was initially identified by comparison of proteins which were labelled with ^{125}I in intact cells grown in iron-replete and in iron-depleted medium. Previously, iodination catalysed by iodobeads and lactoperoxidase has been used successfully to label outer-membrane proteins of the bacteria *Pasteurella multocida* (Lu *et al.*, 1988) and *Vibrio cholerae* (Richardson & Parker, 1985). With *P. haemolytica*, iodobead-catalysed iodination resulted in approximately 20 proteins being reproducibly labelled. Confirmation that outer-membrane proteins were among the proteins iodinated by this procedure comes from labelling of the 70 kDa and 100 kDa IRPs, which are known to reside in the outer membrane. Presumably, some of the periplasmic proteins, including the 35 kDa IRP, are also accessible to the iodination procedure.

The cellular location of the 35 kDa protein was

Fig. 6. Antigenicity of the 35 kDa HPLC-purified protein in SPF lambs. Two SPF lambs were inoculated with partially purified 35 kDa protein preparation on days 0 and 28. Serum samples were taken prior to inoculation on day 0 (pre-immune), and then on days 28, 42 and 57. The development of the immune response was then monitored by immunoblotting using the pooled serum from the two lambs. The antigen sources were as follows: lanes labelled A, whole cells grown in $-Fe$ medium; lanes labelled B, the partially purified 35 kDa protein used for immunization of the lambs.

Fig. 7. The cellular location of the 35 kDa antigen (arrow) as determined by immunoblotting of *P. haemolytica* serotype A2 cell fractions. Cells grown in $-Fe$ medium were fractionated as described in Methods, separated by SDS-PAGE and probed with the anti-35 kDa monoclonal antibody (Ph12/16). Each sample was diluted to a different extent in the fractionation procedure and gel loadings were adjusted to compensate for this. Lanes: 1, lysate of whole cells grown in $-Fe$ medium; 2, insoluble contents of the cytoplasm; 3, soluble contents of the cytoplasm; 4, insoluble contents of the periplasm; 5, soluble contents of the periplasm; 6, outer membranes; 7, cytoplasmic membranes; 8, cell wall envelope.

established by immunoblotting of cellular fractions using the anti-35 kDa monoclonal antibody. This indicated that the 35 kDa protein was located mainly in the periplasmic space. Further support for an intracellular location for the 35 kDa protein was provided by an indirect immunofluorescence test (data not shown) in which the anti-35 kDa monoclonal antibody failed to show any detectable reaction with intact A2 cells grown on $-Fe$ medium. This indicates that the 35 kDa protein, or at least the epitope recognized by the monoclonal antibody, is not expressed at the cell surface.

Evidence is not yet available to establish whether the 35 kDa IRP, together with other previously-identified IRPs, plays any role in engendering the protective immunity found in sheep which have been previously exposed to *P. haemolytica* A2. However, immunoblotting indicated that the 35 kDa protein is strongly expressed by *P. haemolytica* in the animal host *in vivo* (Fig. 5), as

indeed it is on $-Fe$ medium *in vitro* (Fig. 1). Furthermore, the 35 kDa protein is strongly antigenic, since a marked antibody response to the protein is found in animals which have recovered from infection with *P. haemolytica* A2. There is good reason, therefore, to explore the possibility of the involvement of the 35 kDa protein in evoking protection.

At present a function for the 35 kDa protein cannot be proposed. It is unlikely that it is simply a breakdown product of a high molecular mass protein since immunoblotting of the whole cell lysate showed that the anti-35 kDa monoclonal antibody was specific for the 35 kDa antigen, giving only a weak background reaction against other cellular proteins. The possibility that the 35 kDa protein is a stress protein cannot be discounted. However, expression of the 35 kDa protein *in vitro* is unlikely to be attributable to toxic effects of the iron chelator, 2,2'-dipyridyl, or to adverse cultural conditions, since the protein is also synthesized *in vivo*. It is concluded from this that the 35 kDa protein may have a specific metabolic function in *P. haemolytica*. IRPs have been identified in several Gram-negative bacterial species (Neilands, 1982). In some instances these proteins have been shown to have a role in iron uptake whereas others have not known iron-transport function (Payne, 1988).

A 37 kDa IRP has been identified as a major component of the outer membrane of *Neisseria gonorrhoeae* (Mietzner *et al.*, 1984; West & Sparling, 1985) and an antigenically related protein has been found in several other species of *Neisseria* (Mietzner *et al.*, 1986). In *Escherichia coli* two periplasmic proteins have been identified that are involved in iron transport: FepB (molecular mass between 31.5 and 36.5 kDa), which mediates the transport of ferrienterobactin through the cell envelope (Pierce & Earhart, 1986), and FecB (30.8 kDa), which is involved in transport of ferri-citrate complexes (Staudenmaier *et al.*, 1989). It is possible that the 35 kDa protein also has a function in iron uptake or in the transport or release of chelated iron. However, to date no examples of iron-regulated periplasmic proteins have been described in *P. haemolytica* or related species. Further studies are required to determine whether the 35 kDa IRP is involved in uptake or transport of iron in *P. haemolytica*.

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