Association of the P64k dihydrolipoamide dehydrogenase to the Neisseria meningitidis *membrane*

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INVESTIGACIÓN

ABSTRACT

Bacterial dihydrolipoamide dehydrogenases are usually found to be part of the cytoplasmic α-oxoacid dehydrogenase multienzyme complexes. However, our group has been able to isolate a dihydrolipoamide dehydrogenase from *Neisseria meningitidis*, termed P64k, by using polyclonal antisera raised against meningococcal outer membrane proteins.To better understand its biochemical role, protease accessibility, as well as immunoelectromicroscopy techniques were used to study the subcellular localization of P64k. Our results suggest that a significant fraction of the neisserial P64k dihydrolipoamide dehydrogenase is envelope-associated in a compartment sensitive to external proteases, although the low resolution of the methods used so far precludes a more detailed assignment of its location.

Keywords: Neisseria meningitidis, LpdA gene, dihydrolipoamide dehydrogenase, proteín carrier, P64k protein

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RESUMEN

Asociación de la dihidrolipoamida deshidrogenasa (proteína P64k) a la membrana de *Neisseria meningitidis*. Las dihidrolipoamidas deshidrogenasas bacterianas se encuentran usualmente formando parte de los complejos multienzimáticos de las α-cetoácido deshidrogenasas en el citoplasma. Sin embargo, nuestro grupo ha podido aislar la proteína dihidrolipoamida deshidrogenasa de *Neisseria meningitidis* denominada P64k, utilizando sueros policlonales contra proteínas de membrana externa de *N. meningitidis*. Para entender mejor su papel bioquímico, se utilizaron técnicas de susceptibilidad a proteasas y de inmunomicroscopía electrónica para estudiar localización subcelular de la proteína P64k. Nuestros resultados sugieren que una fracción importante de la P64k se encuentra asociada a un compartimiento externo sensible a proteasas, aunque la baja resolución de los métodos empleados hasta ahora no nos permite disponer de una asignación más detallada de su localización.

Palabras Claves: Neisseria meningitidis, gen LpdA, lipoamida deshidrogenasa, proteína portadora, P64k

*I*ntroduction

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) is a flavin-containing pyridine nucleotide disulfide oxidoreductase that catalyzes the NAD^+- or $NADP^+$-dependent oxidation of dihydrolipoamide [1]. It is usually found either as part of the α-oxoacid dehydrogenase multienzyme complexes, where it participates in the oxidative decarboxylation of ketoacids such as pyruvate, α-oxoglutarate, and branched ketoacids resulting from the transamination of branched aliphatic amino acids; or as part of the glycine decarboxylase complex known as the glycine cleavage system [2].

Given the size, structure and function of α-oxoacid dehydrogenase complexes, dihydrolipoamide dehydrogenases are seldom thought of as membrane-associated proteins. However, a growing amount of experimental evidence suggests that they can be at least partially associated as a peripheral protein to the cytosolic membrane in several bacteria, see for example [3-8].

Our group has cloned a dihydrolipoamide dehydrogenase, termed P64k, from the Gram negative bacterium *Neisseria meningitidis* by using polyclonal sera raised against meningococcal outer membrane proteins [9-12]. Given the obvious interest of this result for vaccine development, particularly for its use as a protein carrier in vaccine preparations [13, 14], as well as for the elucidation of the physiological role of P64k if it is in fact found in the outer membrane, a more comprehensive examination of its localization is needed. Here we study, the subcellular localization of the *N. meningitidis* P64k dihydrolipoamide dehydrogenase using different experimental approaches.

*E*xperimental procedures

Bacterial strains and growth conditions

Escherichia coli strain XL-1 Blue [15] was employed for all cloning work. It was grown in a Luria Broth (LB) at 37 ºC. For rRecA and rTbpB expression, strain W3110 [F^- *mcr*A *mcr*B in (*rrn*D-*rrn*E) lambda$^-$] or MM294 F^- *end*A1 *hsd*R17 (r_k^- m_k^+) *sup*E44 *thi*-1 *rel*A1? *rfb*D1? *Spo*T1? transformed with the proper plasmids, were grown in a M9 medium [16, 17] supplemented with 1% glucose, 1% casein hydrolysate and 50 mg/mL ampicillin.

Neisseria meningitidis strain CU B385 (B: 4:P1.19, 15), isolated from a patient with meningococcal disease, was obtained from the Finlay Institute (Havana, Cuba). Strain CU B385Δ*lpdA* is an isogenic *lpdA* (P64k) deletion mutant obtained by electroporation of CU B385 with the suicidal plasmid pM110 (here), under

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previously described conditions [18]. Both strains were grown either in Brain Heart Infusion (BHI, OXOID, UK) agar plates at 37 ºC in a candle jar, or in 5 mL BHI broth cultures at 37 ºC, inoculated to an initial optical density (OD_{620}) of 0.1 and grown for 2 h to the exponential phase.

Reagents and biochemicals

DNA restriction and modification enzymes were purchased from Heber-Biotec (Havana, Cuba). Oligonucleotides were synthesized by standard phosphoramidite chemistry at CIGB. Trypsin from bovine pancreas, TPCK-treated, was obtained from Serva (Heidelberg, Germany). Chymotrypsin (Sequencing grade) was purchased from Sigma (Saint Louis, USA) and pronase E was obtained from Boehringer Mannheim GmbH (Germany). The confirmatory DNA sequence for the constructs pM-180 and pM-181 was done following the Sanger method [19], using the Sequenase 2.0 Kit (USB, USA).

Plasmids

pM110

Plasmid pM110 carries a partially deleted copy of the meningococcal *lpdA* gene, retaining only 100 and 200 bp at its 5' and 3' termini and interrupted by the kanamycin resistance cassette from pUC4K [18]. It was constructed by the digestion of pM3 [12] with *Xho* I and subsequent Exonuclease III/S1 nuclease treatment [20], selecting timepoints with deletions of approximately 1400 bp and ligating the resulting DNA to a blunted Km^r cassette. The deletion size in the plasmids obtained with this procedure was characterized by *Pvu* II restriction analysis.

pM153

Codes for the peripheral outer membrane transferrin receptor (TbpB) from *N. meningitidis* CU B385, as described in [22].

pM181

This plasmid codes for the *N. meningitidis* CU B385 RecA protein as a C-terminal fusion to the first 60 amino acids (aa) of human IL-2, under the control of the *E. coli* tryptophan promoter (ptrp). It was obtained by amplifying the neisserial *recA* gene using the Polymerase Chain Reaction (PCR) under standard conditions, with oligonucleotides 2244 (5' TCTAGACATGAAAATGGACGGCAGC 3') and 3070 (5' CGAATTCAAACGCGCCGTTGTAGC 3'). The purified fragment was used to replace the coding sequence of the meningococcal Opc antigen in plasmid pILM29 [23] by using the *Xba* I and *Bam* HI restriction sites engineered into the amplification primers.

Monoclonal antibodies and sera

Monoclonal antibody (MAb) 5E8 is an IgG2b immunoglobulin that recognizes a linear epitope on the second variable loop of the Class 1 porin (P1) of *N. meningitidis* CU B385. This epitope is surface-exposed on intact cells. This MAb, kindly provided by Dr. Consuelo Nazábal (CIGB, Havana Cuba) recognizes the sequence HYTQNNADVFP corresponding to VR2 serosubtype P1.15 (data pending publication).

Murine MAbs 448/30/7 and 114 (IgG2a) have been described [12, 24]. Both recognize linear and exposed (in the native protein) epitopes on P64k, defined by aa 1-5 and 561-570, respectively of the published sequence (Embl Accession number: X77920). These MAbs do not show cross-reactivity to any other neisserial protein as evaluated by Western Blot and Whole-Cell ELISA using strain CU B385Δ*lpdA* (data not shown). All MAbs used here were previously affinity-purified using ProteinA-Sepharose.

A polyclonal serum against the meningococcal RecA protein expressed in *E. Coli* was obtained as follows: Crude cell extracts from *E. coli* W3110 (pM181) grown as described in this paper were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the band corresponding to overexpressed recombinant RecA (rRecA) was excised with a scalpel, minced, and crushed by passing through a 32-µm metal sieve disk (Carl Schroeter, Germany). The resulting suspension was quantitated by visual comparison with BSA standards run together on 12.5% SDS-PAGE and stained with Coomassie Blue R250. Finally, twenty Balb/c mice were immunized subcutaneously with three doses of 20 mg of the obtained rRecA in complete (first dose) or incomplete (second and third doses) Freund's adjuvant, administered on days 0,7 and 21. Mice were bled at day 28 and the sera were pooled and stored at -20 ºC.

To obtain a polyclonal serum against TbpB from *N. meningitidis* CU B385, strain MM294 was transformed with pM153 and grown as described in obtaining rRecA. The rTbpB was purified from inclusion bodies essentially by cellular disruption using a French press, washing the insoluble fraction with 1% Triton® X-100 and 1 M urea in a 50 mM Tris-Cl buffer pH 7.4, and the extraction of the protein from the resulting precipitate with 6 M guanidinium hydrochloride in the same buffer. This preparation was equilibrated in 50 mM triethanolamine (TEA) buffer pH 9.6 using Sephadex-G25 gel filtration chromatography, and subjected to further purification by ion exchange in MonoQ-Sepharose with a 0-1 M NaCl gradient. The purified protein was used to immunize Balb/c mice as outlined for rRecA, substituting $Al(OH)_3$ for Freund's adjuvant .

The polyclonal serum against P64k was raised by immunizing a New Zealand white rabbit with three doses of 100 mg of P64k purified as previously published [10], in complete (first dose) or incomplete (second and third doses) Freund's adjuvant, following a 0-14-27 immunization schedule. Serum samples were taken 15 days after the third dose.

Preparation of cell extracts

Intact cell extracts were prepared by harvesting exponential cultures of meningococcal strains CU B385 and CU B385Δ*lpdA*, resuspending to an OD_{620} of 4.0 in cold PBS. Disrupted cell extracts were prepared by subjecting aliquots of intact cell extracts to 6 cycles of sonication for 30 sec at full power on a B. Braun Labsonic 2000 sonicator, with 1-min intervals on ice. Both intact- and disrupted-cell extracts were prepared fresh for each experiment and immediately used.

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Immuno-transmission electron microscopy (ITEM)

The analysis of samples by ITEM was carried out as described [25]. Briefly, *N. meningitidis* CU B385 and CU B385Δ*lpdA* intact cell extracts were fixed with 0.2% glutaraldehyde/, 4% paraformaldehyde in PBS, dehydrated in ethanol, embedded in Araldite resin (Fluka, Switzerland), and polymerized at 70 °C. Ultrathin sections (400-500 Å) were cut on a NOVA microtome (LKB, Germany), collected on nickel 400 mesh grids, and immersed for 15 min in blocking buffer (1% bovine serum albumin, 0.02 M glycine in phosphate-buffered saline, pH 7.3), followed by incubation for 45 min with MAbs at 0.2 mg/mL, or with mouse or rabbit polyclonal sera diluted 1:10 in blocking buffer. After three rinses for 30 min in washing buffer (0.1% bovine serum albumin in phosphate-buffered saline, pH 7.3), the sections were incubated for 1 h at room temperature in Protein A-colloidal gold (PAG) conjugate (15 nm particle size, Heber-Biotec, Cuba) diluted 1:100 in blocking buffer, and rinsed again three times for 30 min in washing buffer. After drying, the sections on grids were stained for 5 min in 7% uranyl acetate and 7 min in 0.25% lead citrate before being examined under a JEOL/JEM 2000EX transmission electron microscope. The specificity of the detection was assessed by including controls with an unrelated MAb and by using the PAG conjugate alone.

Quantitation of cytoplasm –and cell periphery– associated label was done by counting the gold particles in 10 randomly selected cells from the same section using the software DIGIPAT (Eicisoft, La Habana, Cuba). The statistical processing of results was carried out using a Kruskal-Wallis non-parametric ANOVA as implemented in the software GraphPad Prism™ (GraphPad Software Inc., San Diego CA, USA).

Determination of protease sensitivity in intact cells

Four hundred microliters of intact or disrupted cell extracts were incubated alone or with decreasing amounts (3.12, 1.56, 0.78 and 0.39 mg) of trypsin, chymotrypsin or pronase E for 12 h at 4 °C. The samples were then spun at 12 000 x g for 5 min at 4 °C, resuspended in 100 mL Laemmli sample buffer, and heated to 95 °C for 5 min to stop the reaction.

Ten microliters of each sample for each protease were subjected to 12.5% SDS-PAGE, immunoblotted onto 0.45 mm nitrocellulose membranes, and probed with MAbs or sera against Class 1, rTbpB, P64k and rRecA. Immunodetection was carried out as follows: the membranes were blocked by incubating them with 5% skimmed milk powder in PBS containing 0.1% (v/v) Tween® 20 [blocking solution (PBS-T)] for 1 h at room temperature (RT) and washing once with PBS-T, followed by an incubation overnight at 4 ºC with the MAbs 5E8 (class 1), 114 (anti-P64k) or 448/30/7 (anti-P64k) at 5 mg/mL, or rTbpB-specific (1:1000) or rRecA-specific (1:400) antisera, diluted in the blocking solution. The following morning the membranes were washe of three times with PBS –T and incubated for 1 h at RT with either anti– mouse or anti-rabbit IgG peroxidase-linked whole antibody (Amersham plc. UK) diluted 1:3000 in the blocking solution. After three washes in PBS-T the signal was detected by chemiluminiscence using the ECL Western blotting kit from Amersham plc.

Results and discussion

Localization of P64k by ITEM

In an effort to study the subcellular localization of the *N. meningitidis* P64k dihydrolipoamide dehydrogenase, ultrathin sections from exponentially growing cells were examined by transmission electron microscopy after being subjected to immunodetection with antibodies against P64k, comparing them to sections treated with antibodies against the class 1 protein (P1), one of the major porins on the meningococcal outer membrane [26], or the cytoplasmic RecA protein.

Figures 1 A show the results of the immunodetection when using MAb 5E8 against P1 in the CU B385Δ*lpdA* mutant (the same results were obtained with CU B385 –data not shown–). It is observed that, the label is conspicuously associated with the cell periphery in both strains, which also confirms that there are no gross changes in the subcellular localization in the mutant. As expected, a more detailed assignation of P1 to the periplasmic space or either the inner or outer membrane is impossible, since the actual gold particle can be found up to 12 nm away from the actual site of the antigen due to the size of the PAG complex [27]. On the other hand, the labeling pattern is clearly cytoplasmic when incubating the samples with mice polyclonal sera against rRecA (Figure 1, B and C). As expected, in both strains the label is distributed evenly throughout the cytoplasm, with some particles showing up in the cell periphery due to the low resolution of the technique.

Figures 1D and E show the results of the immunodetection of P64k with MAb 114 against P64k. Although most of the label is found on the cytosol, there is clearly a specific labeling of the cell periphery in strain CU B385. This labeling is specific for P64k, since it is not found on the P64k knockout strain CU B385Δ*lpdA*. The same result was obtained with the P64k MAb 448/30/7 (data not shown) and when using rabbit polyclonal sera against P64k (Figures 1F and G). However when using the latter there is some labeling on strain CU B385Δ*lpdA*, the pattern is cytoplasmic, and it is probably due to cross reactivity with either the dihydrolipoamide dehydrogenase from the α-oxoglutarate dehydrogenase complex (unpublished results) or the E2 component from the pyruvate dehydrogenase complex [28].

The above results were confirmed by analyzing the data quantitatively. The gold particles from 10 cells chosen randomly from each section were counted and assigned either to the cytoplasm or to the cell periphery, then the fraction of the total counts, which was cell periphery-associated, was compared between groups using a non-parametric ANOVA. Figure 2 shows in the dispersion diagram the percent of the label associated with the cell envelope in each case. For strain CU B385 with MAb 114, 29.9% (SD 9.6%) of the gold particles were located on the cell periphery, which was not statistically different ($p<0.05$) from the 21.3% (SD 6.5%) found on the same strain when using the anti-P64k polyclonal serum. However, both values differ significantly ($p<0.05$) from those found

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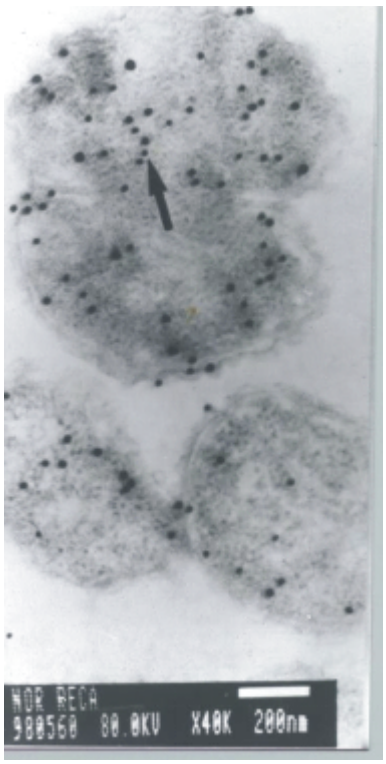
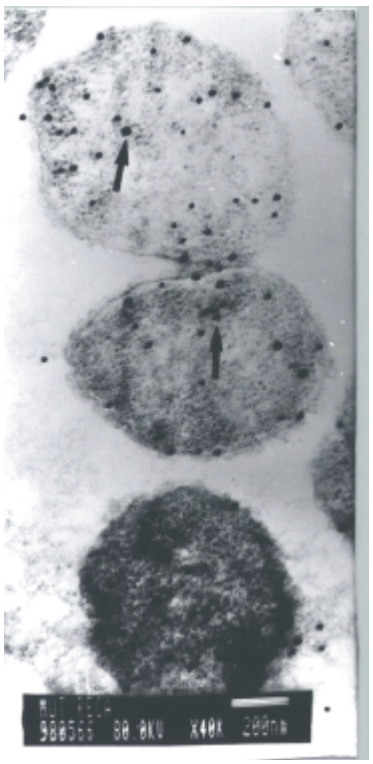
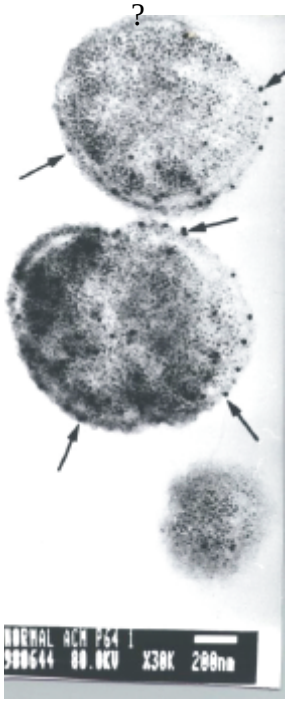
1A. B385Δ*lpdA* with mAb 5E8



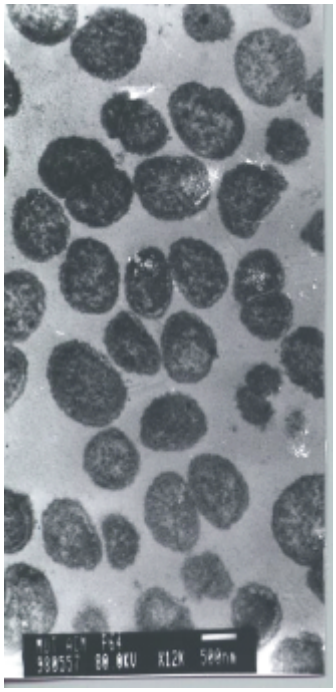
1B. B385 with anti/RecA



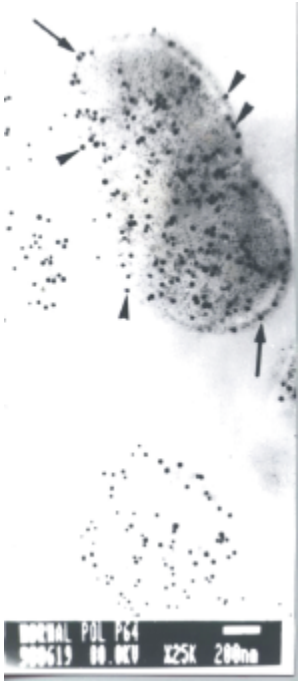
1C. B385Δ*lpdA* with anti/RecA



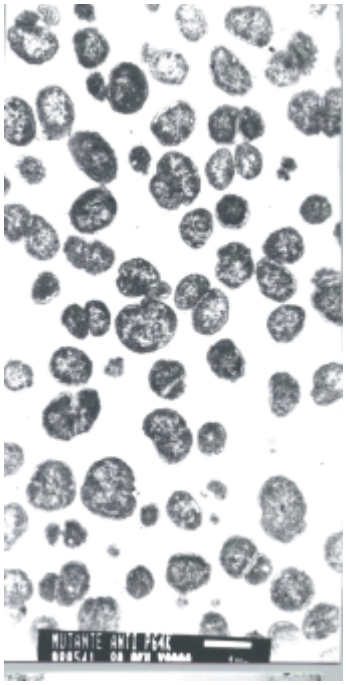
1D. B385 with mAb 114



1E. B385Δ*lpdA* with mAb 114



1F. B385 with anti-P64k polyclonal



1G. B385Δ*lpdA* with anti-P64k polyclonal

Figure 1. Immunoelectromicroscopy of CU B385 and CU B385Δ*lpdA N*. meningitidis strains. The bar represents XX nm. A: CU B385 incubated with mAb 5E8 (anti-P1) x 200; B: CU B385Δ*lpdA* with mAb 5E8 (anti-P1) x 200 nm; C: CU B385 with anti-rRecA polyclonal sera x 200 nm; D: CU B385Δ*lpdA* with anti-rRecA polyclonal sera x 200 nm; E: CU B385 with mAb 114 x 200 nm; F: CU B385Δ*lpdA* with mAb 114 x 500 nm. G: CU B385 with anti-P64k polyclonal serum x 200 nm; H: CU B385Δ*lpdA* with anti-P64k polyclonal serum x 1μ.

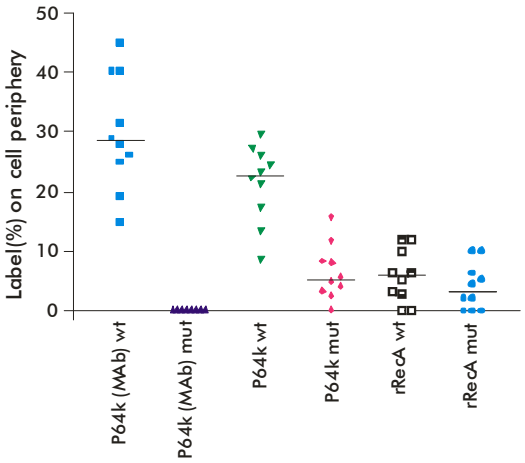


Figure 2. Percent of Protein A-colloidal gold complexes associated with the cell periphery. Each point represents the counts for one cell within one group, and the horizontal line marks the median for each group. P64k (mAb) wt: mAb 114 on CU B385. P64k (mAb) mut: mAb 114 on CU B385Δ*lpdA*. P64k wt: Rabbit polyclonal serum against P64k on CU B385. P64k mut: Rabbit polyclonal serum against P64k on CU B385Δ*lpdA*. rRecA wt: Mice polyclonal sera against rRecA on B385. rRecA mut: Mice polyclonal sera against rRecA on B385Δ*lpdA*.

when using the anti-P64k polyclonal serum on CU B385Δ*lpdA* (6.4%, SD 4.7%) or the anti-rRecA polyclonal sera on either strain (5.7%, SD 4.4% for CU B385, 4.0% SD 3.9% for CU B385Δ*lpdA*). Furthermore, there were no statistically significant differences between the three latter samples, confirming that the mixed periphery/cytoplasm labeling pattern found when using anti-P64k serum on the wild type switches to a cytoplasmic pattern when the *lpdA* gene is eliminated by mutagenesis.

Protease sensitivity of P64k in intact cells

Given that immunoelectromicroscopy does not allow the unequivocal assignment of P64k to a specific compartment of the cell envelope, a series of experiments was carried out to determine if it was accessible to exogenous proteases in intact cells. To this end, intact cell extracts were subjected to proteolytic treatment, the reaction stopped, and the integrity of P64k, RecA, and the peripheral outer membrane transferrin receptor TbpB was assessed by Western bloting.

Figure 3 A shows the immunodetection of P64k with MAb 448//30/7 on cells subjected to decreasing concentrations of chymotrypsin (the same results were obtained with trypsin and pronase E –data not shown–). It can be seen that a large fraction of the cellular P64k is, in fact, accessible to external proteases. This degradation is not due to cellular proteolysis, as no degradation is noticeable on control lanes without added protease and furthermore, it is dependent on the amount of protease added. Also, it can not be ascribed either to the tendency of meningococci to autolyse [29], or to the loss of cellular integrity due to the proteolytic treatment, since no degradation is detected when using the same samples to immunodetect a cytoplasmic protein like RecA (Figure 3 C). On the other hand, the resistance of RecA and part of the cellular P64k is not due to protease-resistant conformations, since both proteins are completely sensitive to proteolytic degradation once cellular integrity is eliminated by ultrasonic disruption (Figures 3 B and D). Lastly, under these experimental conditions TbpB behaves like a typical outer membrane protein, in that it is protease-sensitive irrespective of cellular integrity (Figures 3 E and F).

The results presented here strongly suggest that the meningococcal P64k dihydrolipoamide dehy-drogenase is located both in the cytoplasm and in a protease-accessible compartment of the cell-envelope. This

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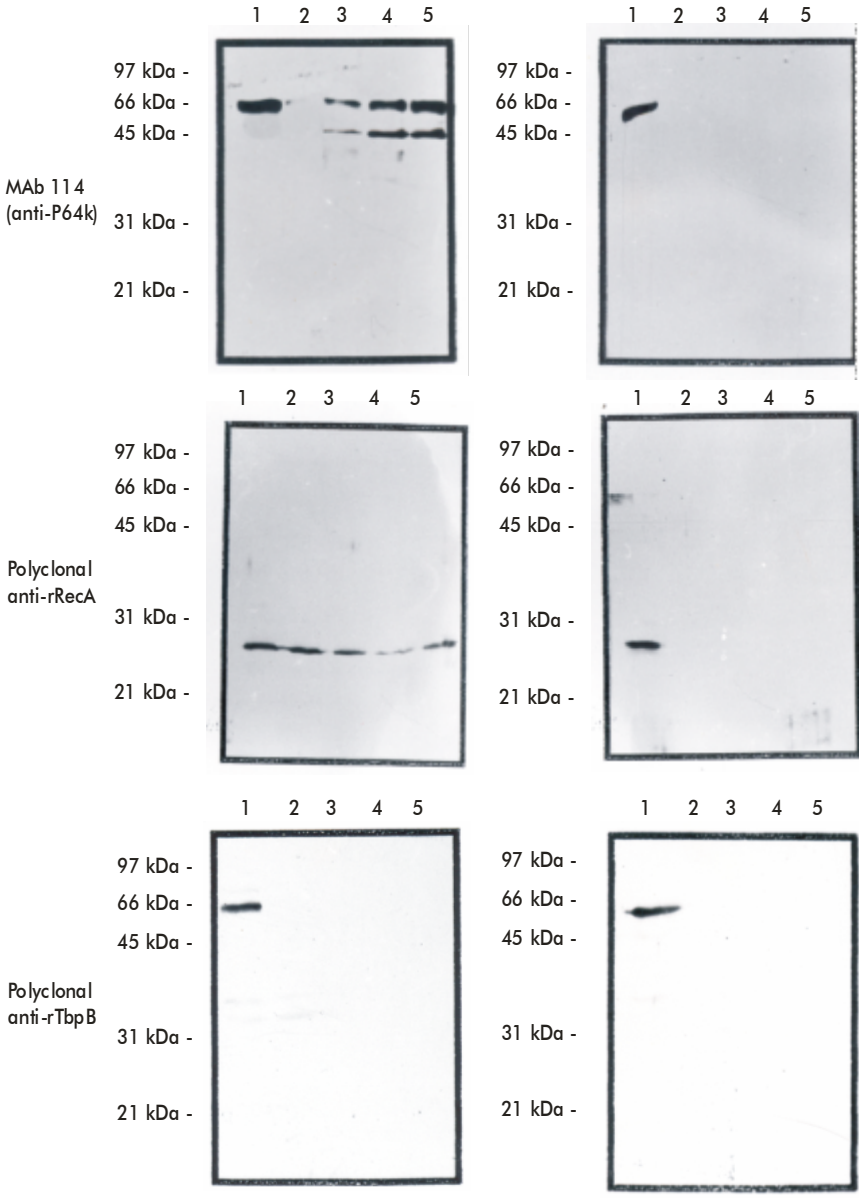

Figure 3. Western blotting of intact and disrupted cell extracts subjected to decreasing concentrations of chymotrypsin. For all panels, lane 1: No protease; lane 2: 3.12 g of chymotrypsin; lane 3: 1.56 g of chymotrypsin; lane 4: 0.78 g of chymotrypsin; lane 5: 0.39 g of chymotrypsin. Left panels (A, C, E): Intact cell extracts; right panels (B, D, F): disrupted cell extracts. The proteins detected are P64k (Top row, A and B), RecA (middle row, C and D) and TbpB (bottom row, E and F).

conclusion is based both on a mixed peripheral/ cytoplasmic labeling pattern during immunoelectromicroscopy of ultrathin sections, and in the presence of protease-sensitive and protease-resistant fractions in intact cells, in complete agreement with the above results. Although based on protease sensitivity alone the envelope-associated fraction of P64k does not seem to be associated with the cytoplasmic side of the inner membrane. The reported leakyness of the meningococcal outer membrane [30], together with the lack of a peri-plasmic control in our experiments, precludes a more detailed assignment of its location within the cell envelope.

It is difficult to envisage a function for such an envelope-associated dihydrolipoamide dehydrogenase. Dihydrolipoamide dehydrogenase has been found in archaebacteria and in the mammalian bloodstream form of *Trypanosoma brucei*, where neither α-oxoacid complexes nor glycine cleavage systems are found [31, 32]. This fact evidences that it may fulfill additional, as yet undiscovered cellular roles, and has led to the proposal that it has been recruited during evolution from another function to serve in such complexes [31]. Also, dihydrolipoamide dehydrogenase has been suggested or proved to be envelope-associated in a variety of eubacteria [3-7, 34], in eukaryotic mitochondriae [11, 35], and trypanosomatid protozoa [30]. In this context it is worth noting that the dithiol-disulphide exchanges typical of dihydrolipoamide dehydrogenase catalysis might be ideally suited for oxidation-reduction reactions involved in membrane transport and signal transduction, and in fact, the presence in *E. coli* of lipoic acid-dependent transport systems which are inhibitable by dithiol-specific arsenicals has been reported [30, 36]. However, given the absence of NAD^+ and $NADP^+$ outside the cytosol, questions about the role of a dihydrolipoamide dehydrogenase like P64k are hard to answer. Our results suggest that a significant fraction of the neisserial P64k dihydrolipoamide dehydrogenase is envelope-associated in a compartment sensitive to external proteases, although the low resolution of the methods used so far precludes a more detailed assignment of its location.

These results will lead to a better understanding of the physiological role of this protein on the bacterial surface and ultimately on the possibilities of its use as a protein carrier in vaccine immunization of different weak immunogens. Although P64k itself is far from being considered a promising candidate antigen for protein-based anti-meningococcal vaccines, its capability to stimulat a memory response in humans, especially at a low dose, has encouraged us to continue using this meningococcal antigen as a potential protein carrier in future conjugated vaccines [13, 14] . Usually, for human vaccines, haptens have been coupled to TT or diphtheria toxoid, because these proteins are commercially available and have been used in humans for a long time without side-effects [37]. However, concern is increasing regarding the epitope overload and possible suppression when the same molecule is used in several vaccines [38, 39]. P64k could be a new alternative, considering the limited availability of protein carriers and the need for developing new conjugate vaccines. The T-cell epitopes in this meningococcal protein are currently being investigated. A Phase I clinical study demonstrated that the meningococcal recombinant P64k is safe and immunogenic in humans upon immunisation [40], and therefore, the use of this antigen as a protein carrier in future conjugate vaccines is feasible and should be exploited.

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