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Multiple SecA Protein Isoforms in *Escherichia coli*

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To define the anti-SecA-LacZ antiserum, immunoprecipitates produced with either whole anti-SecA-LacZ rabbit antiserum or affinity-purified antibodies were used to analyze nondenatured lysates of *Escherichia coli*. The antiserum contains antibodies that recognize different proteins. Antibody purified by preadsorption to the SecA-LacZ hybrid protein precipitated only the SecA protein from extracts. In contrast, antibody purified from the intact SecA protein precipitated several additional proteins with SecA protein. Ribosomal protein L_7L_{12} is one of the polypeptides coprecipitated with SecA protein by antibody purified by immunoadsorption to the intact SecA protein as well as by unfractionated anti-SecA-LacZ antiserum. Two-dimensional gel electrophoresis of the SecA protein immunoprecipitated by either antiserum or purified antibody indicated that the SecA protein exists in at least two, and probably four, isoforms. Only one of the SecA isoforms is present in a ribosomal preparation.

The *Escherichia coli secA* gene produces an essential component of the secretion machinery that is responsible for the proper localization of many outer membrane and periplasmic proteins (14–16). The *secA* gene product, a 92-kilodalton (kDa) protein, has been identified by precipitation of heat- and detergent-denatured cell extracts with an antiserum produced by immunization of a rabbit with purified SecA-LacZ fusion protein (15). Using this same antiserum, I have immunoprecipitated the SecA protein from nondenatured cell extracts. In this report, the immunoprecipitated proteins were analyzed by isoelectric focusing (IEF) and sodium dodecyl sulfate (SDS) two-dimensional (2D) gel electrophoresis because the method resolves the majority of *E. coli* proteins (13). The SecA protein was located on the 2D profile of total proteins, and its protein neighbors were identified. The data also show that the SecA protein of *E. coli* consists of several isoforms.

MATERIALS AND METHODS

Antisera, protein sources, and ribosomes. The anti-SecA-LacZ antiserum was previously characterized (15) and was kindly provided by D. Oliver. A variety of other rabbit antisera against various *E. coli* proteins, used to identify antibody specificities that are common to many sera, were obtained as follows: anti-GroEL, R. Hendrix; anti-β-galactosidase, A. Fowler; three anti-Rho antibodies, R. Grant; anti-L_7L_{12}, M. Nomura; anti-resolvase, B. Newman; anti-Klenow fragment, C. Joyce; and anti-fc goat immunoglobulin G was commercially obtained from Cooper Biomedical, Inc. Four nonimmune sera were provided by J. Rodman, and rabbit immunoglobulin G was obtained commercially from Sigma Chemical Co. Purified DNA polymerase I was kindly provided by C. Joyce, and thioredoxin was graciously given by R. Grant. RNA polymerase was purified by the Burgess and Jendrisak procedure (1). A ribosomal preparation that had been purified through a 0.5 M KCl–2 M sucrose cushion (8) was provided by P. Moore.

Strains and medium. MC4100, MM52, and the *secA-lacZ* gene fusion on the pF1 plasmid were from D. Oliver and have been described (16). CGSC 5689 from B. Bachmann, containing the K18 *aroP-lpd* deletion (5), was used as the donor for transducing the deletion into MM52 by selecting for $secA^+$ and screening for the simultaneous requirement for acetate and succinate in minimal medium. The resultant strain produced for this study, MC4100 Δ(*aroP-lpd*)*18* $secA^+$, is isogenic with MC4100 and is CGSC 6766. FB8 is a wild-type $lacZ^+$ *E. coli* K-12 strain that was provided by A. Danchin.

Cells were grown in synthetic minimal medium [0.1 M KCl, 0.05 M Tris, 0.8 mM $(NH_4)_2SO_4$, 0.5 mM PO_4, 1 mM $MgSO_4$, 0.1 mM $FeCl_3$, pH 7.5] that was supplemented with 0.4% glucose, vitamins, and all amino acids at 0.2 mM except methionine. When necessary for growth of the *aroP-lpd* deletion strain, acetate and succinate (10 mM) were provided. Cells were grown by shaking at 37°C. [^{35}S]methionine was provided at a concentration of 50 μCi/ml in the presence of 1 μg of unlabeled methionine for analytical samples or with 5 μCi/ml for preparative samples. Cells were harvested by centrifugation 20 min after the label had been added.

Immunoprecipitation of native extracts. Cell pellets from 1 ml of culture were suspended in 20 μl of 20% sucrose–30 mM Tris (pH 7.5) and incubated for 5 to 7 min on ice. A 5-μl sample of a solution of lysozyme (10 mg/ml) in 0.1 M EDTA was added. After 5 to 7 min, 25 μl of 30 mM Tris (pH 7.5)–0.05% Nonidet P-40–20 mM NaCl was added to lyse the cells. The lysate was centrifuged in the cold with an Eppendorf centrifuge for 5 min. The supernatant was used as the cell lysate for all experiments. The EDTA present in the second solution is used to chelate the many divalent ions present in the lipopolysaccharide layer of the outer membrane and is therefore not available for chelating metals in the cell supernatant after lysis occurs by the addition of the third solution. The EDTA chelation of metals of the lipopolysaccharide layer allows lysozyme access to the peptidoglycan layer to destabilize the outer envelope.

Protein A-Sepharose CLB (Pharmacia Fine Chemicals) was hydrated in NET2 buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 0.05% Nonidet P-40) and incubated for 2 to 5 h with antiserum or purified antibody. The beads were washed two times in NET2 buffer,and all liquid was removed. The beads were then incubated with lysate for 20 min minimum on ice and were washed five times in NET2 buffer. They were then mixed with O'Farrell IEF lysis buffer and subjected to either Laemmli SDS-gel electrophoresis or O'Farrell 2D gel electrophoresis (13). After electrophoresis,, the gels were stained in 0.1% Coomassie blue–5% methanol–7.5% acetic acid, destained, rinsed with water several times, and incu-

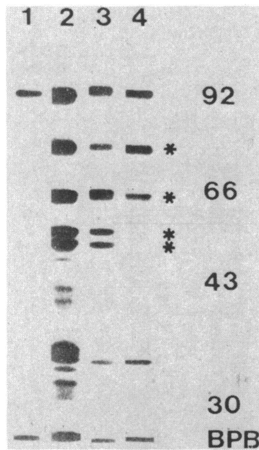

FIG. 1. Immunoprecipitation of [35S]methionine-labeled lysate from 10^8 cells of MC4100, a *lacZ* deletion strain, with 2 μl of rabbit anti-SecA-LacZ antiserum. After washing of the immunprecipitates, the samples were incubated in SDS sample buffer at 100°C for 2 min, run on a 7.5% acrylamide–SDS resolving gel, and fluorographed. The positions and sizes of molecular size standards (kDa) are indicated to the right, as is the position of the bromphenol blue dye front (BPB). The asterisks represent proteins which are commonly immunoprecipitated by many immune rabbit sera. In lane 1, the lysate was boiled in 1% SDS, diluted 10-fold into 1% Triton–50 mM Tris–10 mM NaCl, immunoprecipitated, and washed as described in Materials and Methods. In lane 2, the nondenatured lysate was immunoprecipitated and washed as described in Materials and Methods. In lane 3, the immunoprecipitate was prepared as for lane 2, but was washed two times with 1 M NaCl–50 mM Tris–1% Triton. In lane 4, the immunoprecipitate was prepared as for lane 2, but was washed two times with 50 mM Tris–0.1% SDS–1% Triton and then two times with NET2 buffer. The slight shift in mobility of the SecA protein in lane 3 is due to the presence of Triton; additional washes of the immunoprecipitate with NET2 did not change the quantity or type of precipitated proteins, but did downshift slightly the position of the SecA protein to that of lanes 1, 2, and 4.

bated with 0.5 M sodium salicylate for at least 30 min. The gels were dried and autoradiographed.

Affinity purification of different antibodies from the antiserum. Affinity purification by immunoadsorption required at least three pure proteins: the SecA-LacZ hybrid protein, β-galactosidase (which is the *lacZ* gene product), and the intact SecA protein. To obtain the SecA-LacZ hybrid protein and the β-galactosidase protein, a [35S]methionine-labeled lysate of either the MC4100 pF1 strain or the isopropyl-β-D-thiogalactopyranoside-induced FB8 strain, which respectively contain the SecA-LacZ hybrid protein or β-galactosidase, was incubated with rabbit anti-β-galactosidase antiserum. To obtain the intact SecA protein, a [35S]methionine-labeled MC4100 lysate was incubated with 10 times the amount of anti-SecA-LacZ antiserum that was used for Fig. 1, lane 2. All three of the preparative immunoprecipitates contained more than one protein, but contaminating species migrated with different mobilities than the proteins of interest. The three pure proteins were obtained after electrophoresis of the immunoprecipitates on an SDS-polyacrylamide gel and transfer to activated diazobenzylmethyl (DBM)-paper.

35S-proteins were transferred from SDS gels to activated DBM-paper as described by Olmsted (17) and the Schleicher & Schuell Co. protein blotting manual. An autoradiograph of the DBM-bound transferred antigens was used to identify the SecA-LacZ hybrid protein, the β-galactosidase protein, or the SecA protein. The DBM sheets, containing all of the transferred proteins from the three preparative immunoprecipitates, were cut into pieces. Different antibodies were then purified by immunoadsorption to each piece of the transfer, as well as to the pure SecA-LacZ hybrid protein, β-galactosidase protein, or intact SecA protein. Antibodies purified from the β-galactosidase protein, the GroEL protein, the L_7L_{12} protein, or a blank sample served as controls.

Each small strip containing either the protein of interest or a blank strip was incubated with either anti-SecA-LacZ antiserum or anti-β-galactosidase antiserum. The strips were extensively washed in NET2 buffer, and the adsorbed antibody was eluted with 4 M guanidinium chloride in phosphate-buffered saline as suggested by J. G. Howe (6). The antibodies were immediately dialyzed against phosphate-buffered saline, incubated with protein A-Sepharose beads, and used to immunoprecipitate cell lysates of MC4100.

RESULTS

Anti-SecA-LacZ antiserum immunoprecipitates several proteins from nondenatured cell lysates. The *secA* gene product was previously defined as the protein precipitated from boiled, SDS-denatured cell lysates made from an *E. coli lacZ* deletion strain by antiserum raised against a SecA-LacZ hybrid protein (3, 15, 16). That antiserum was produced in a rabbit immunized with a hybrid protein containing the first third of the SecA protein fused to the *lacZ* gene product, β-galactosidase; it is used here to characterize the SecA protein in nondenaturing conditions. Since the culture medium, the immunoprecipitation method, and the source of protein A used in the immunoprecipitation were different from those in the previous reports, the precipitation from denatured and nondenatured lysates was compared to repeat the initial findings. The [35S]methionine-labeled cells grown in the medium used here were denatured with heat and SDS, diluted into a Triton buffer, and immunoprecipitated by the Protein A-sepharose technique described in Materials and Methods. A single major SecA protein species of 92 kDa was precipitated (Fig. 1, lane 1), duplicating the previously published findings (3, 15, 16). However, when a nondenatured cell lysate was used as the source of antigen, many additional proteins were present in the immunoprecipitates (lane 2). Several of the proteins, but not SecA, were selectively removed when the precipitates were washed with either 1 M NaCl (lane 3) or 0.1% SDS (lane 4).

The presence of additional proteins in the immunoprecipitates from the nondenatured extracts could result from the following: (i) occurrence of multiple antibody specificities in the antiserum, (ii) immunoprecipitation of a protein complex in which only a single protein is antigenic (24), or (iii) nonspecific immunoprecipitation of other polypeptides. Unfortunately, preimmune serum from the rabbit was not available to help reconcile these possibilities. A comparison between commercial nonimmune rabbit immunoglobulin G and anti-SecA-LacZ antiserum indicated that immunoprecipitation by nonimmune rabbit immunoglobulin G was negligible (data not shown). When the immunoprecipitates of 14 different antisera (described in Materials and Methods) were compared, it was obvious that the four proteins labeled with an asterisk in Fig. 1 were present in the majority (data not shown). This result suggested that there may be several antibodies that recognize different proteins in the anti-SecA-LacZ antiserum, and some might be common to many sera.

Preparation of protein fractions used to purify antibodies. To determine whether there were multiple antibody speci-

ficities in the anti-SecA-LacZ antiserum, I purified all of the antibodies present in the antiserum that would bind to DBM-bound antigens. The number and type of proteins that were subsequently immunoprecipitated from nondenatured cell extracts characterized each isolated antibody fraction. The method used to purify the antibodies (17) was affinity immunoadsorption to isolated proteins that had been electrophoresed onto DBM-paper as described in Materials and Methods. The proteins used for the affinity purification had to be pure to obtain monospecific antibody fractions. I required the original antigen, the SecA-LacZ fusion protein, to isolate the mixture of anti-SecA and anti-β-galactosidase antibodies that would uniquely bind to it. I also needed β-galactosidase to use as an affinity adsorbant for isolating only the monospecific β-galactosidase antibodies that would serve as a control. Both the β-galactosidase protein and the SecA-LacZ fusion protein were isolated either from isopropyl-β-D-thiogalactopyranoside-induced FB8 extracts or from MC4100 pF1 cell extracts. These proteins were purified by immunoprecipitation with a rabbit antiserum that had been raised against native purified β-galactosidase protein. Analytical [^{35}S]methionine-labeled samples of these immunoprecipitates (Fig. 2, lanes 2 and 4, respectively) were compared with their respective total crude extracts (Fig. 2, lanes 1 and 3). Both the β-galactosidase protein and the SecA-LacZ fusion protein were large proteins (115 and 165 kDa, respectively). There were no proteins which comigrated with β-galactosidase (compare lanes 1 and 2 to lane 3). The only proteins which migrated near the SecA-LacZ fusion protein in crude extracts were the B and B′ subunits of RNA polymerase. These two proteins were not present in the anti-β-galactosidase immunoprecipitates (compare lanes 1 and 2). Thus the only very large protein present in lane 4 is the SecA-LacZ hybrid fusion protein. However, because other smaller proteins were present in the precipitates, preparative samples were first fractionated by SDS-polyacrylamide gel electrophoresis. The electrophoresed proteins were transferred to DBM-paper and localized by autoradiography. The different DBM fragments containing the pure SecA-LacZ fusion protein and the β-galactosidase protein were then used to affinity purify antibodies from the anti-SecA-LacZ antiserum. Alternative fragments containing other proteins or a blank piece of DBM-paper were used as controls.

An abundant source of pure intact SecA protein was not available. Therefore, a preparative immunoprecipitate produced by anti-SecA-LacZ antiserum was electrophoresed and transferred to DBM paper to produce the intact SecA protein. After autoradiography of the DBM sheet, the portion containing only the SecA 92-kDa region was obtained. Other successive fragments of the sheet were also used as controls.

Multiple antibody specificities can be purified from the anti-SecA-LacZ antiserum. One could conclude that multiple antibody specificities are present in the anti-SecA-LacZ antiserum if the various antibody fractions, affinity purified from different fragments of the DBM sheets, precipitated different proteins. I therefore affinity purified the antibodies from the anti-SecA-LacZ antiserum as described in Materials and Methods by using the fragments of DBM-paper described above. The proteins that were precipitated from crude cell extracts by the affinity-purified antibodies are shown in Fig. 3. Fig. 3A displays the proteins precipitated by antibodies that were purified from anti-SecA-LacZ antiserum by using DBM-papers bound with either the intact SecA protein (lane 2) or other fractionated antigens of a

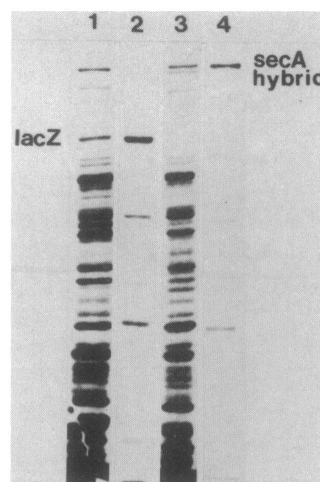

FIG. 2. Isolation of the pure SecA-LacZ hybrid protein and the β-galactosidase protein. The lysate of the isopropyl-β-D-thiogalactopyranoside-induced LacZ$^+$ FB8 strain (lane 1) or MC4100pF1, the SecA-LacZ hybrid protein containing strain (lane 3), are displayed on a 6% acrylamide–SDS gel fluorogram. A 1-μl sample of anti-B-galactosidase serum was used to immunoprecipitate 5×10^8 cells of FB8 (lane 2) or MC4100pF1 (lane 4). The position of β-galactosidase, the *lacZ* gene product, and the SecA-LacZ hybrid protein are indicated. Duplicate β-galactosidase immunoprecipitates were prepared with 100 μl of antiserum and approximately 2×10^{10} cells to produce the β-galactosidase or SecA-LacZ hybrid protein required for affinity purification of antibody fractions.

preparative anti-SecA-LacZ antiserum sample (lanes 3 through 8). The profiles can be compared to the immunoprecipitates produced by antibodies isolated from a blank DBM piece (lane 1), by the total anti-SecA-LacZ antiserum (lane 9), by affinity-purified anti-L_7L_{12} antibody (lane 10), or by antibodies purified from anti-SecA-LacZ antiserum by using immunoadsorption to either the pure SecA-LacZ hybrid protein (lane 11) or the pure β-galactosidase protein (lane 12) described above.

In Fig. 3B, other antibodies were purified from anti-SecA-LacZ antiserum (lanes 1 through 5) or from anti-β-galactosidase antiserum (lanes 6 through 10) by immunoadsorption to the other proteins in the β-galactosidase precipitate of MC4100 pF1 that are smaller than the SecA-LacZ hybrid protein. Thus all of the antibodies that can be purified by immunoadsorption to various antigens that were covalently attached to the different DBM fragments were isolated. These include the antibodies found in both the anti-SecA-LacZ antiserum (Fig. 3A, lanes 1 through 7, 11, and 12; Fig. 3B, lanes 1 through 5) and anti-β-galactosidase antiserum, which serves as a control (Fig. 3B, lanes 6 through 10).

Both the anti-SecA-LacZ antiserum and the anti-β-galactosidase antiserum contained multiple antibody specificities (Fig. 3). Only the anti-SecA-LacZ antiserum contained antibody that recognized the 92-kDa SecA protein covalently bound to the DBM paper. One antibody specificity was present in both sera (Fig. 3A, lane 4; Fig. 3B, lanes 3 and 8). This antibody precipitated the GroEL protein (as determined by IEF-2D polyacrylamide gel electrophoresis and comigration with the GroEL protein produced by anti-GroEL antiserum, data not shown).

Even though the antisera contained multiple antibody specificities, antisera could be fractionated to yield mono-

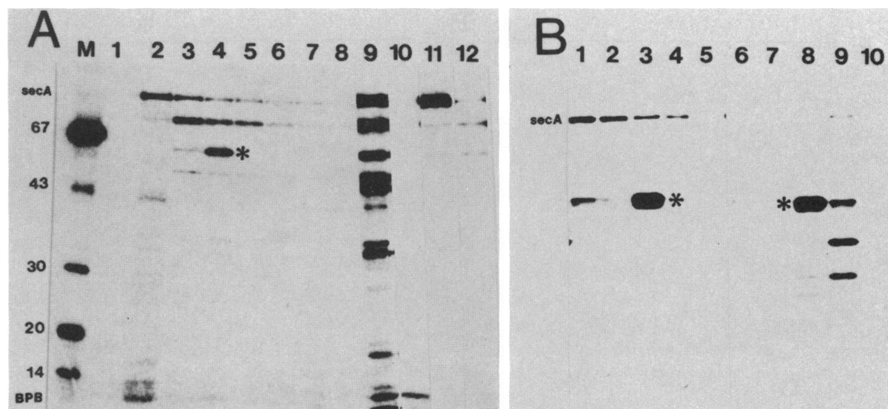

FIG. 3. Immunoprecipitation of crude cell lysates with specific affinity-purified antibody fractions. The intact SecA protein, the SecA-LacZ hybrid protein, the β-galactosidase protein, and other smaller proteins in preparative immunoprecipitates were covalently attached to DBM-papers after electrophoresis as described in Materials and Methods. Different anti-SecA-LacZ antiserum fractions were purified by immunoadsorption to and elution from those DBM-papers. The antibody fractions were then used to prepare immunoprecipitates to define the antibody specificities. The immunoprecipitates shown here were prepared from MC4100 lysates as described in Materials and Methods. The samples were electrophoresed on 7 to 15% acrylamide–SDS gradient gels (A) or 10% acrylamide–SDS gels (B). (A) ^{125}I-labeled protein standards from Pharmacia are present in lane M. Antibody was purified from a blank fragment (lane 1) or from the intact SecA protein (lane 2). In lanes 3 through 8, antibody was purified from successive DBM fragments containing DBM-bound antigens in the preparative anti-SecA-LacZ immunoprecipitate as follows: 3, 90 to 73 kDa; 4, 70 to 60 kDa; 5, 50 to 40 kDa; 6, 40 to 30 kDa; 7, 30 to 20 kDa; 8, 20 to 14 kDa. Lane 9 is the unfractionated anti-SecA-LacZ antiserum immunoprecipitate and lane 10 is the immunoprecipitate of affinity purified anti-L_7L_{12} polyclonal antibody. Next are the immunoprecipitates of antibody purified by adsorption to either the pure SecA-LacZ hybrid protein (lane 11) or the pure β-galactosidase protein (lane 12). (B) Antibody was purified from either anti-SecA-LacZ antiserum (lanes 1 through 5) or anti-β-galactosidase antiserum (lanes 6 through 10) by adsorption to DBM-papers containing antigens that were present in the preparative MC4100 pF1 precipitate used to produce the pure SecA-LacZ hybrid protein as follows: 1 and 6, 140 to 100 kDa; 2 and 7, 100 to 70 kDa; 3 and 8, 70 to 55 kDa; 4 and 9, 55 to 35 kDa; 5 and 10, controls that used a blank DBM sheet. The asterisks identify the GroEL protein.

specific antibodies that could be used for selective immunoprecipitation of specific proteins (Fig. 3). Thus antibody can be isolated that immunoprecipitates only the SecA protein (Fig. 3A, lane 11), the GroEL protein (Fig. 3A, lane 4; Fig. 3B, lanes 3 and 8), the L_7L_{12} protein (subsequently identified by IEF-2D gel electrophoresis; data not shown) (Fig. 3A, lane 10), or the β-galactosidase protein (antibody identical to that used for Fig. 3A, lane 12, if the extract is *lacZ*$^+$; data not shown).

When a blank DBM piece was used for the purification (Fig. 3A, lane 1), or when the antigen for a certain antibody was not present in the extract (Fig. 3A, lane 12, for the β-galactosidase protein), essentially no protein was present in the subsequent immunoprecipitates. In addition, when an antigen was present on the DBM sheet, but no antibody specific for it was in the antiserum to be fractionated, then none of that protein was found in the subsequent immunoprecipitates (compare Fig. 3B, lanes 4 and 9, which are equivalent DBM pieces but different antisera). These last two findings strongly indicate that nonspecific immunoprecipitation was minimal, even with mild wash conditions.

A comparison of the data of Fig. 1 and 3 suggests that the state of the antigen (denatured or nondenatured) affects whether it is recognized by the antibody. Several antibody-antigen complexes were resistant to detergent and high salt. These findings show that affinity purification of antibody and subsequent immunoprecipitation from nondenatured crude extracts provides a reliable purification method for the analysis of individual proteins or their corresponding antibodies. Moreover, they prove that the anti-SecA-LacZ antiserum has many antibody specificities to various different proteins, including the primary antigens (the SecA and β-galactosidase proteins) which were presented as a fusion protein.

One surprising finding was the coprecipitation of a number of minor proteins with the SecA protein by the antibody purified by affinity adsorption to the intact SecA protein (Fig. 3A, lane 2). These minor proteins are also found in precipitates of whole anti-SecA-LacZ antiserum (Fig. 3A, lane 9) (identities were verified by comigration after 2D gel electrophoresis; see Fig. 5). Their absence in the blank control (Fig. 3A, lane 1) or in the immunoprecipitates of antibody-purified by binding to either the β-galactosidase protein (Fig. 3A, lane 12) or the SecA-LacZ hybrid protein (Fig. 3A, lane 11), suggests that they associate selectively with a form of the SecA protein that is recognized by distinct antibody species. Those antibody species are isolated only by immunoadsorption to the intact SecA protein and not to the SecA-LacZ hybrid protein.

Identification of multiple SecA protein isoforms by 2D gel electrophoresis. It was desirable to define the immunoprecipitated SecA protein by 2D gel electrophoresis. As described above, purified antibody fractions had been isolated that immunoprecipitate only the SecA protein by one-dimensional SDS-polyacrylamide gel electrophoresis. Those antibodies were obtained by affinity immunoadsorption to the pure SecA-LacZ hybrid protein (Fig. 3A, lane 11). The extract was prepared from a *lacZ* deletion strain and contained essentially no protein precipitated by the β-galactosidase-specific antibodies. Therefore, the polypeptides present must be precipitated by the only other antibodies present-those to the SecA protein. The only polypeptides present in this sample after 2D gel electrophoresis were resolved into two major charged species of 92 kDa which represent the isoforms of SecA (Fig. 4A). Two minor

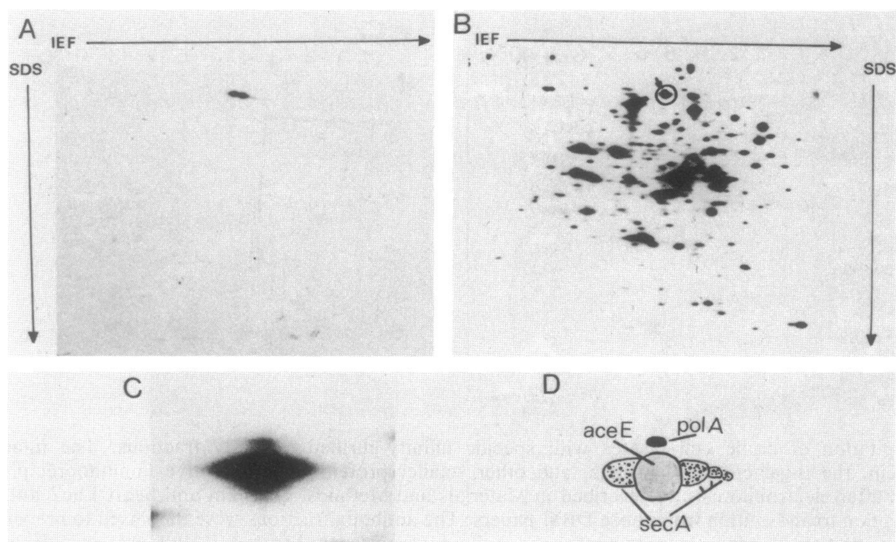

FIG. 4. Multiple isoforms of SecA can be resolved by 2D gel electrophoresis. Pure SecA protein was prepared as described for Fig. 3A, lane 11, with antibody that was affinity purified by adsorption to the pure SecA-LacZ hybrid protein. A 4-μg sample of RNA polymerase holoenzyme was added to the [^{35}S]methionine-labeled SecA sample (A) and to a sample of MC4100 [^{35}S]methionine-labeled lysate (B). All samples were electrophoresed (13), stained and fluorographed. The stained proteins were used to align the different autoradiograms to determine the position of the SecA protein in the lysate (circle in panel B). This region has been magnified in panel C. Comparison with the gene-protein index (12) and other published catalogs of *E. coli* proteins allowed the identification of the proteins surrounding SecA in the circle as shown in panel D.

charged SecA 92-kDa species were apparent in longer autoradiographic exposures. These identical SecA protein isoforms were present in immunoprecipitates produced with either total anti-SecA-LacZ antiserum (Fig. 5) or antibody that was purified by immunoadsorption to the intact SecA protein (data not shown).

The polypeptide pattern of the immunoprecipitate was then compared and aligned with the MC4100 lysate pattern. Before electrophoresis, the [^{35}S]methionine-labeled samples were mixed with sufficient RNA polymerase holoenzyme to provide visible markers for alignment of the different autoradiograms. The holoenzyme subunits provide four known markers of different size and charge (4). Figure 4B displays all of the *E. coli* lysate proteins that fractionate in the pH 3 to 10 range. The position of SecA protein in the lysate was determined by aligning the RNA polymerase markers in the total pattern with those in the immunoprecipitates. Thus, the SecA protein migrated in a distinctive region that contained a very limited number of proteins (Fig. 4B, circle).

The 2D polypeptide patterns were compared with those in the gene-protein index (12) and with other published patterns (4, 9, 22) to determine whether any of the proteins in the circled region had been previously identified or analyzed. Using the published patterns, it became apparent that the highest-molecular-weight peptide in the circled region was DNA polymerase I, the product of the *polA* gene. The highly abundant protein which migrated between the two major SecA isoforms appeared to be the large subunit of pyruvate dehydrogenase (the pdhE1 protein of Smith and Neidhardt [22]), which is the product of the *aceE* gene. Using the data produced by different autoradiographic exposures of the numerous samples that were examined, as well as published patterns, it was possible to construct the diagram of Fig. 4D, which illustrates the relationship of the proteins circled in Fig. 4B and C (the AceE, PolA, and SecA proteins) to each other.

In the autoradiogram shown in Fig. 4B, the radiation from the abundant *aceE* gene product exposes a larger area than the protein actually corresponds to, as determined by making timed exposures. Because of this, the AceE spot partially obscured the less abundant SecA protein isoforms in the autoradiograms of the *E. coli* lysate of Fig. 4B. Therefore, an isogenic derivative of MC4100, strain CGSC 6766, was constructed which contained the *aroP-lpd* K18 deletion (5) as described in Materials and Methods. Since the *aceE* gene is included in this deletion, extracts of the newly constructed strain should be missing the abundant protein spot, which migrates between the major SecA isoforms. The isogenic derivative was desired because the 2D patterns produced by different strains can vary. I also added purified DNA polymerase I as a marker protein to radioactive MC4100 and CGSC 6766 extracts and to the immunoprecipitate of anti-SecA-LacZ antiserum and compared the 2D gel electropherograms produced (data not shown). The marker DNA polymerase I protein and the missing pdhE1 protein, as compared with the position of SecA in the antiserum precipitate, verified the identification and position of the SecA protein as depicted in Fig. 4B, C, and D.

The physical heterogeneity of the SecA protein is not an artifact of sample preparation. Although a number of proteins in *E. coli* are known to have multiple isoforms, often as a result of posttranslational modification (i.e., IF2 [18], L_7L_{12} [21], S_6 [20], and others [12]), artifactual multiple isoforms can be generated during preparation of the samples or during electrophoresis in either dimension because of urea carbamylation (13). The last possibility seemed remote because the total protein pattern produced with the extracts (Fig. 4B) looked normal. It seemed unlikely that the process of immunoprecipitation would artifactually create isoforms because the solutions contained only ingredients (0.05% Nonidet P-40, 150 mM NaCl, and Tris) that are present in higher concentrations in the IEF solutions and that would not be expected to alter the charge of polypeptides.

To rule out the possibility that modifications occurred

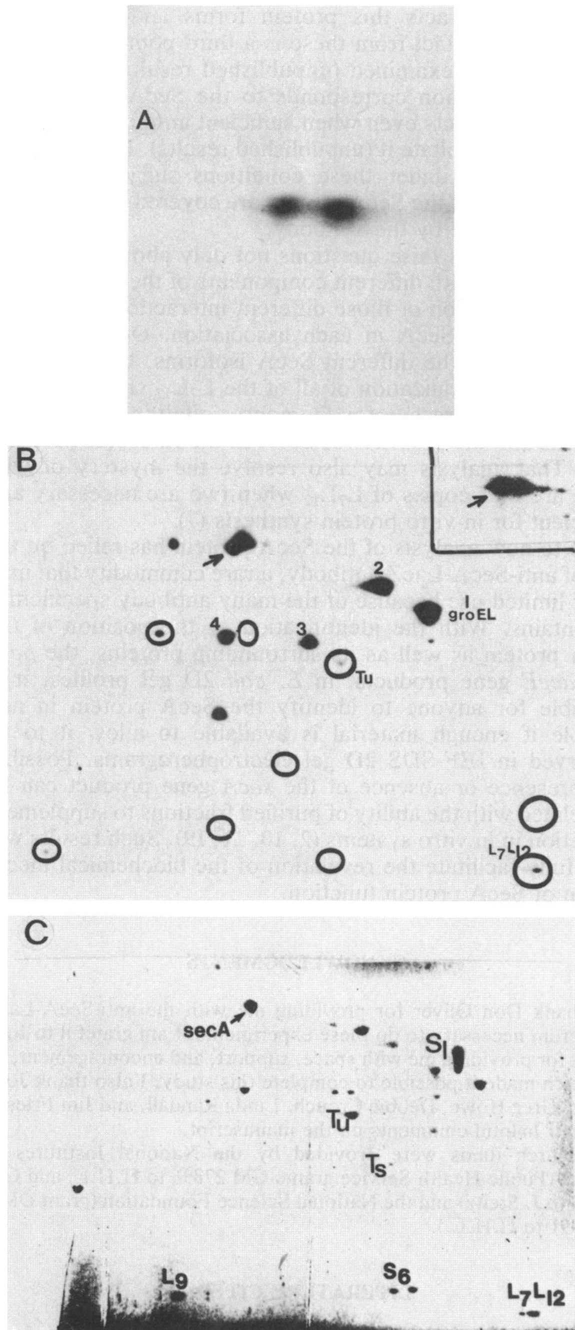

FIG. 5. Multiple SecA isoforms are not an artifact of sample preparation. An anti-SecA-LacZ antiserum immunoprecipitate was prepared by using a lysate that was first denatured with 1% SDS and heat before being diluted 10-fold into 1% Triton (A). Alternatively, an immunoprecipitate from a nondenatured extract was used (B, inset). A nonradioactive 2-μl sample of a ribosomal preparation (purified with 0.5 M KCl through a 2 M sucrose cushion [8]; optical density at 260 nm of 960) was added to the nondenatured immunoprecipitate before 2D gel electrophoresis (12). The resulting gel was stained with Coomassie blue before destaining and fluorography. A fluorogram shows the radioactive immunoprecipitated proteins in panel B; the stained proteins with a pI below 10 that are present in the ribosomal preparation are shown in panel C. A comparable immunoprecipitate was electrophoresed with no ribosomal protein addition; no stained proteins were present. All labeled proteins were identical in migration in the duplicate sample; only the region of the Sec A isoforms are shown in panel A and the inset to panel B. The arrow indicates the position of SecA in C and its isoforms in B and the inset to B; the nonradioactive SecA in the ribosomal preparation overloads the basic SecA radioactive isoform, causing it to move slightly more rapidly in the second dimension. The numbers refer to the proteins commonly found in many antisera which were identified with asterisks in Fig. 1. The positions of the ribosomal proteins are indicated. The circles represent the proteins which are found in addition to SecA in immunoprecipitates produced by antibody that was affinity purified by immunoadsorption to the intact SecA protein as described for Fig. 3, lane 2.

after lysis, lysates were prepared with 1% SDS followed by heat at 100°C for 3 min. After a 10-fold dilution into 1% Triton, immunoprecipitates were made as described previously and subjected to IEF-SDS 20 electrophoresis. Two SecA protein isoforms were apparent (Fig. 5A), suggesting that the charge differences between the two isoforms are stable to both heat and detergents and are not due to modifications during or after the lysis procedure used to generate either nondenatured or denatured immunoprecipitates.

The possibility of artifact generated during electrophoresis could be ruled out if a single isoform of SecA maintained its unity in a sample containing several isoforms. Surprisingly, it became apparent that a ribosomal preparation (prepared in the conventional method with 0.5 M KCl and a 2 M sucrose cushion [8]) included a single isoform of the SecA protein. The imunoprecipitate of the anti-SecA-LacZ antiserum was combined with a sample of the ribosomal preparation (Fig. 5B and C, respectively). The combined samples were then electrophoresed to resolve the acidic ribosomal proteins. The immunoprecipitate did not contain sufficient protein to be stained, as determined from a duplicate sample that was run by itself. The 2D profile of the labeled proteins of both samples of this immunoprecipitate could be aligned with the other; therefore, only the SecA region of the sample that did not contain ribosomes is shown in the inset. The resulting autoradiograms (Fig. 5B and inset) were compared with the stained protein profile of the gel, which resolved numerous ribosomal proteins including S_1, EFTu, EFTs, L_9, S_6, and L_7L_{12} (Fig. 5C). The stained SecA protein in the ribosomal preparation (arrow, Fig. 5C) comigrated exactly with the most basic SecA isoform in the immunoprecipitate (Fig. 5B). The SecA that was in the ribosomes slightly changed the mobility of the basic SecA [^{35}S]methionine-labeled isoform spot (Fig. 5B and inset). However, the absolute alignment of the stained SecA protein with the radioactive isoform in the mixed sample proves that they are identical. Therefore, the multiple forms of the SecA protein are not an artifact of sample preparation, because the single isoform of SecA from the ribosomal preparation maintains its unity in the mixed sample, whereas the radioactive SecA resolves into multiple isoforms. The SecA isoform spots immunoprecipitated with unfractionated anti-SecA-LacZ antiserum (Fig. 5B) are identical to the pure SecA isoforms (Fig. 4A) that are precipitated by antibody purified by immunoadsorption to the pure SecA-LacZ hybrid protein.

The identity of the small 12-kDa protein present in the anti-SecA-LacZ antiserum immunoprecipitate is also clarified in this experiment. This small protein is the ribosomal protein L_7L_{12}, since both the radioactive protein and the stained L_7L_{12} comigrated exactly in the same 2D gel. Ribosomal protein L_7L_{12} was also immunoprecipitated by antibody that was purified by adsorption to the intact SecA protein (immunoprecipitate of Fig. 3A, lane 2, and its 2D gel

electropherogram compared with the immunoprecipitate of affinity-purified anti-L_7L_{12} antibody, Fig. 3A, lane 10, and its 2D gel electropherogram; data not shown). Moreover, when SecA-LacZ fusion protein was purified by two methods that recognized the unique β-galactosidase moiety of the fusion (immunoprecipitation and affinity chromatography [23]), ribosomal protein L_7L_{12} was also present (unpublished results). Significantly, comparable preparations of β-galactosidase tested by the same methods lacked the ribosomal protein. Thus, two different technical procedures, immunoprecipitation with antiserum or column affinity chromatography, result in copurification of the ribosomal protein L_7L_{12} and the SecA protein.

DISCUSSION

The technique of immunoprecipitation of nondenatured cell extracts with anti-SecA-LacZ antiserum or with antibodies that were affinity purified from it is sufficiently stringent to allow the identification of an individual protein species from all the other proteins in a crude extract. Using this technique, I have demonstrated that the SecA protein found in nondenatured *E. coli* extracts exists in at least two, and perhaps four, isoforms that can be identified by fractionation of immunoprecipitates on IEF-SDS 2D gels. At present, the reason for the charge heterogeneity of the SecA protein is not known, but analysis of total ^{32}P-labeled *E. coli* proteins shows that SecA is not phosphorylated (unpublished results). The intriguing question of whether the multiple SecA isoforms are separately localized within the cell can be raised, because only one of the charged isoforms of SecA was found in ribosomal preparations. This suggests that determining the biochemical function of the SecA protein in in vitro protein synthesis systems may prove difficult since one source of SecA protein is provided by the necessary addition of ribosomes to the system.

I have demonstrated that if the proper antibody is used, SecA can be coprecipitated with a selective protein set, including the ribosomal protein L_7L_{12}. It is possible that the coprecipitation of proteins with SecA by affinity-purified antibody (Fig. 3) is the result of isolating a complex containing SecA associated with other proteins. The ability to remove those proteins with high salt and SDS (Fig. 1) supports this suggestion. The alternative possibility, that the purified antibody recognizes the same determinant on the SecA protein, the L_7L_{12} protein, and the other six coprecipitated proteins, is improbable. Moreover, I have purified L_7L_{12} polyclonal antibodies by immunoadsorption to the L_7L_{12} protein and find that they do not precipitate SecA. Thus, L_7L_{12} does not share antigenic determinants with SecA. The most likely conclusion that can be drawn from the results presented herein is that some fraction of the SecA polypeptides is able to associate with various proteins, including the ribosomal protein L_7L_{12}. That form of SecA is recognized by only some antibodies in the serum.

The SecA protein isoforms may exist in at least two populations in crude, nondenatured extracts. The first population corresponds to the SecA protein that was coprecipitated with several other proteins by an antibody that was purified by immunoadsorption to the intact SecA protein. The coprecipitation of these proteins suggest that they associate to form a complexed SecA population, in which the SecA antigenic sites are still available to the antibody. The second population corresponds to the pure SecA protein that is obtained by precipitation with antibody that was purified by immunoadsorption to the SecA-LacZ hybrid protein. In extracts this protein forms an uncomplexed population. Distinct from these is a third population that is currently being examined (unpublished results). This inaccessible population corresponds to the SecA protein that remains in extracts even when sufficient antibody is present to immunoprecipitate it (unpublished results). Its inability to be precipitated under these conditions suggests that the antigenic sites of the SecA protein are covered and therefore not recognizable by the antibody.

These findings raise questions not only about the association of SecA with different components of the cell, but also about the function of those different interactions and about the amount of SecA in each association. Obviously, the quantitation of the different SecA isoforms, their localization, and the localization of all of the L_7L_{12} should provide insight into the reason for the coprecipitation of these two proteins and the presence of SecA in the ribosome preparation. That analysis may also resolve the mystery of why there are four copies of L_7L_{12} when two are necessary and sufficient for in vitro protein synthesis (7).

Up to now analysis of the SecA protein has relied on the use of anti-SecA-LacZ antibody, a rare commodity that may be of limited use because of the many antibody specificities it contains. With the identification of the position of the SecA protein as well as its surrounding proteins, the *polA* and *aceE* gene products, in *E. coli* 2D gel profiles, it is possible for anyone to identify the SecA protein in any sample if enough material is available to allow it to be observed in IEF-SDS 2D gel electropherograms. Possibly the presence or absence of the *secA* gene product can be correlated with the ability of purified fractions to supplement secretion in in vitro systems (2, 10, 11, 19). Such results will hopefully facilitate the revelation of the biochemical mechanism of SecA protein function.

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