Calcium-dependent Conformational Changes in the 36-kDa Subunit of Intestinal Protein I Related to the Cellular 36-kDa Target of Rous Sarcoma Virus Tyrosine Kinase*

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Protein I from intestinal epithelium is biochemically and immunologically related to the fibroblast 36-kDa substrate of the Rous sarcoma virus-encoded tyrosine protein kinase (Gerke and Weber (1984) EMBO J. 3, 227–233). Protein I is a Ca2+-binding protein containing two copies each of a 36- and 10-kDa subunit. Denaturation/reconstitution experiments show that the 36-kDa subunit is a monomer, whereas the 10-kDa subunit forms a dimer. Mixing of the subunits leads to reconstituted protein I. Physicochemical properties of protein I and its isolated subunits reveal a Ca2+-dependent conformational change in the 36-kDa subunit which involves the exposure of 1 or more tyrosine residues to a more aqueous environment. This change points to a Ca2+ binding constant of about 104 M–1 in the presence of 2 mM Mg2+ and induces the ability of protein I and the 36-kDa subunit to bind in vitro to F-actin and nonerythroid spectrin. The same high Ca2+ requirement has been reported for the in vitro tyrosine phosphorylation of a 35-kDa protein from A-431 carcinoma cells by the epidermal growth factor receptor kinase (Fava and Cohen (1984) J. Biol. Chem. 259, 2636–2645). Here we show that this 35-kDa substrate is biochemically and immunologically related to the 36-kDa subunit of protein I, which in turn corresponds to the subunit of the Rous sarcoma virus kinase. The protein of A-431 cells exists not only as a monomer but also as a dimer. The latter fraction contains a 10-kDa polypeptide immunologically related to the corresponding subunit of protein I. Given past results on the A-431 system, we speculate that the monomer rather than the dimer is the preferred in vitro substrate for the epidermal growth factor receptor kinase. Thus, the 10-kDa subunit, which induces dimerization of the phosphorylatable large subunit, may act as an inhibitor.

Avian sarcoma viruses encode transforming proteins that appear to function as tyrosine-specific protein kinases (for review see Ref. 1). Among the cellular targets of these kinases is a relatively basic protein whose polypeptide molecular weight is around 34–39,000, designated below as 36,000 (2–11). This protein which can be isolated in small amounts from chick embryo fibroblasts (4, 12) is phosphorylated in vitro by Rous sarcoma virus protein kinase (4).

We have shown that a 36-kDa subunit, immunologically related to the fibroblastic 36-kDa kinase substrate, can be readily isolated in large quantities from porcine intestinal epithelium (13). This finding was recently confirmed by others (14, 15) who demonstrated the presence of high levels of the 36-kDa tyrosine kinase substrate in intestinal epithelial cells by immunological criteria. In these highly differentiated cells, the 36-kDa polypeptide was localized by means of immunofluorescence microscopy in the terminal web that underlies the microvilli (13, 15). The 36-kDa distribution in intestinal epithelial cells as well as in fibroblasts (7–11, 13, 15) is similar to that of nonerythroid spectrin (16–19). When isolated from intestinal epithelium, the 36-kDa polypeptide occurs as a complex with a 10-kDa polypeptide (13). The whole complex, referred to as protein I, contains two copies of the 36-kDa subunit. Protein I binds in vitro to F-actin and nonerythroid spectrin in a Ca2+-dependent manner, once the free concentration of the bivalent cation exceeds 50 μM (13).

One approach to understanding the molecular mechanism of sarcoma virus transformation is to begin to study the functions of the target proteins of the tyrosine-specific viral protein kinase. In the case of the 36-kDa protein from chicken fibroblasts, this was difficult since only limited amounts can be isolated. The ability to isolate large quantities of protein I from intestinal epithelium now allows a detailed analysis. Here we characterize some biochemical properties of the 36- and 10-kDa subunit of protein I in the separated state as well as in the complex. Reconstitution experiments show that the dimeric character of the 36-kDa polypeptide is only maintained in the presence of the 10-kDa subunit. The 10-kDa subunit forms a dimer under native conditions. Exposure to Ca2+ induces a conformational change both in protein I and in its 36-kDa subunit. This molecular change involves the exposure of 1 or more tyrosine residues to an aqueous environment. We show also that a 35-kDa protein, recently identified as a target of the EGF1 receptor tyrosine kinase in human epidermoid carcinoma A-431 cells (20), seems closely related but is not identical to protein I.

MATERIALS AND METHODS

Isolation of Protein I and Separation of Subunits—Protein I was isolated from porcine intestinal epithelium as described previously (13). Protein I was dissociated into subunits by dialysis against 9 M urea, 100 mM NaCl, 1 mM EGTA, 2 mM NaN3, 3 mM DTT, in 20 mM Tris- HCl, pH 7.5, and subjected at room temperature to gel filtration through Sephacryl S-200 (Pharmacia, 0.9 × 100 cm) equilibrated in the same buffer. Fractions containing the separated 36- and 10-kDa polypeptide were pooled and individually dialyzed against 100 mM NaCl, 1.5 mM NaN3, 2 mM MgCl2, 0.5 mM DTT, in 20 mM Tris-HCl, pH 7.5, in order to renature the subunits. Subunits could be

1 The abbreviations used are: EGF, epidermal growth factor; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N''-tetraacetate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Rs, Stokes radius.
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stored in renaturation buffer at 4 °C for at least 3 weeks.

Tryptic Peptide Analysis of 10-kDa Subunit—The 10-kDa subunit was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and oxidized with performic acid. Protein was then suspended in 0.1 M ammonium bicarbonate at a concentration of 0.5–1 mg/ml. Tosylsulfonylphenylalanyl chloromethyl ketone-treated trypsin (Serva) was added at an enzyme/substrate ratio of 1% (w/w) and digestion was performed for 6 h at 37 °C. The reaction was stopped by lyophilization. The lyophilized mixture was dissolved in pH 6.5 buffer (10% pyridine, 0.5% acetic acid), clarified by centrifugation, and applied to a Sephadryl S-200 column (0.9×100 cm) packed with Sephacryl S-200 (Pharmacia). The column was equilibrated in 100 mM NaCl, 2 mM NaN₃, 0.5 mM DTT, 1 mM EDTA, 2 mM MgCl₂, 0.1 mM EGTA, 0.1 mM DTT, 2 mM MgCl₂, 0.1 mM EGTA, 50 mM Tris-HCl, pH 7.5, and adjusted to a concentration of approximately 1 mg/ml with the same buffer. Identical 0.75-ml aliquots of these solutions were placed on one side of each cuvette. In the reference cell 0.75 ml of buffer plus 2 mM CaCl₂ were present. After a base-line was obtained, the solutions were mixed well, whereas in the sample cell 0.75 ml of buffer plus 2 mM CaCl₂ were present. The absorbance at 230 nm. Chemical cross-linking of the renatured 10-kDa subunit was performed by adding 5, 10, or 20 μl of freshly prepared dimethyl suberimidate in 0.01 M 2-mercaptoethanol, 0.1 M Na phosphate, pH 8.0 (10 mg/ml), to 30 μl of the 10-kDa polypeptide (0.6 mg/ml) dialedyzed into the same buffer. Incubation was carried out for 2 h at room temperature. The reaction was stopped by addition of 30 μl of 1 M Tris acetate, pH 8.0. After 10 min, protein was precipitated by trichloroacetic acid, washed with acetone, and dissolved in sample buffer for SDS-gel electrophoresis. Podrin from pig brain and porcine muscle actin were isolated as before. Their Ca²⁺-dependent interaction with protein I and its subunits was analyzed as previously described (13).

RESULTS

Separation of the Subunits of Protein I—We have previously demonstrated that protein I from intestinal epithelial cells contains two different subunits with apparent molecular weights of 36,000 and 10,000 when analyzed by SDS-polyacrylamide gels. Given the molecular weight difference, we attempted to separate the two subunits in urea-containing buffers by gel filtration on Sephacryl S-200. No separation was obtained in 6 M urea, indicating a relatively strong interaction. However, raising the urea concentration to 9 M led to a complete dissociation of protein I, and the two polypeptides were easily separated (Fig. 1). Dialysis of the separated subunits against urea-free buffers led to renaturation as judged by the different physicochemical properties described below. Subunits were characterized by their amino acid composition. The results in Table I represent the average of several amino acid analyses and differ slightly from our preliminary calculations which were based on only one analysis (13). The lack of tryptophan in the 10-kDa subunit was confirmed by the UV absorption spectrum which is dominated by the phenylalanine bands (Fig. 2) since the 10-kDa polypeptide contains 8 phenylalanine and only 2 tyrosine residues (Table I). The UV spectra of protein I and the 36-kDa subunit show characteristic tryptophan and tyrosine absorptions which obscure the phenylalanine signals (Fig. 2).
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### Table 1

Amino acid composition of the protein I subunits

| Amino acid | 36-kDa subunit | 10-kDa subunit |
|------------|----------------|----------------|
|            | mol %          | mol %          |
| Cys        | 1.6            | 2.2            |
| Asp        | 11.1           | 10.5           |
| Thr        | 5.1            | 4.0            |
| Ser        | 8.9            | 3.2            |
| Glu        | 12.3           | 12.8           |
| Pro        | 2.9            | 3.7            |
| Gly        | 6.2            | 6.6            |
| Ala        | 7.0            | 5.9            |
| Val        | 4.5            | 3.9            |
| Met        | 2.9            | 7.1            |
| Ile        | 4.6            | 2.9            |
| Leu        | 9.2            | 7.9            |
| Tyr        | 4.6            | 2.2            |
| Phe        | 3.4            | 9.1            |
| Lys        | 9.5            | 12.8           |
| His        | 1.1            | 3.1            |
| Arg        | 5.1            | 2.3            |
| Trp        | 0.3            | 0.0            |

**Fig. 2.** UV absorption spectra of protein I and its subunits. Spectra of protein I (---), 36 kDa (--), and 10 kDa (-----) in 80 mM NaCl, 0.1 mM DTT, 0.1 mM EGTA, and 50 mM Tris-HCl, pH 7.5.

**Molecular Size of the Renatured Subunits and Reassembly of Protein I**—Analytical ultracentrifugation (data not shown) and gel filtration of the renatured 36-kDa subunit provided a molecular mass of 36,000 ± 2,000 and a Stokes radius of 23 ± 1 Å (Fig. 3A). Neither value was influenced by the presence or absence of Ca\textsuperscript{2+}. Gel filtration of the renatured 10-kDa subunit revealed a Stokes radius of 19 ± 1 Å, indicative of a molecular weight of about 20,000 for a globular protein (Fig. 3A). Analytical gel filtration was also performed under denaturing conditions in the presence of 6 M guanidine hydrochloride (23). In such experiments, the 10-kDa subunit eluted at a position corresponding to a molecular weight of 10,000, when compared to other polypeptides of known molecular weight with a random coil conformation (Fig. 3B).

A polypeptide molecular weight of about 10,000 for the small subunit was also obtained by peptide analysis. The tryptic pattern shows 12 peptides (Fig. 4). The amino acid composition and the amino-terminal residue of the peptides are given in the figure legend. In addition to these peptides, some material (T\textsubscript{x}), insoluble at pH 6.5, remains after digestion. Since the 10-kDa subunit shows an amino-terminal proline residue, T\textsubscript{x} is identified as the amino-terminal tryptic peptide. Amino acid sequence data show that T\textsubscript{10} (Phe-Ala-Gly-Asp-Lys-Gly-Tyr-Leu-Thr-Lys) overlaps T\textsubscript{5} (Gly-Tyr-Leu-Thr-Lys) and T\textsubscript{9} (Phe-Ala-Gly-Asp-Lys). The partial amino acid sequence also indicates that T\textsubscript{8} (Gln-Phe-Pro-Gly-Val-Leu-(Asp, Asp, Gln, Ala, Met, Ile, Lys)-Lys) and T\textsubscript{10} (Gln-Phe-Pro-Gly-Val-Leu-(Asp, Asp, Gln, Ala)-Lys) are related peptides. More importantly, there are only 2 arginine-containing peptides and these are clearly unique in sequence.

**Fig. 3.** Determination of Stokes radii and molecular weight by analytical gel filtration and chemical cross-linking of the 10-kDa subunit. Renatured 36- and 10-kDa subunits were subjected to analytical gel filtration on Sephacryl S-200 (Pharmacia) in 100 mM NaCl, 2 mM NaN\textsubscript{3}, 0.5 mM DTT, 1 mM EGTA, and 20 mMimidazole-HCl, pH 7.4, in order to evaluate their Stokes radii under native conditions. Subunits were either chromatographed separately or after mixing at equimolar ratios (36 K + 10 K). Chromatography was on a calibrated S-200 column (A). Note the reconstitution of intact protein I when the 36- and 10-kDa polypeptides were mixed prior to gel filtration (Rs of 41 Å). Molecular weight determination of the 10-kDa subunit by gel filtration in 6 M guanidine HCl on a calibrated S-200 column (Pharmacia, 0.9 × 100 cm) is shown in B. Bovine pancreatic trypsin inhibitor is abbreviated as BPTI. Cross-linking of the renatured 10-kDa subunit was performed with dimethyl suberimide as described under "Materials and Methods." SDS-polyacrylamide gels (7.5–15% acrylamide) of samples incubated with different amounts of cross-linker are shown in lanes D–F and compared to untreated 10-kDa polypeptide (lane C). Note the increase in the formation of the dimer band (∼20,000) with increasing concentrations of dimethyl suberimidate (D–F). BSA, bovine serum albumin.
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**Fig. 4.** Tryptic peptides of the 10-kDa subunit. The subunit was subjected to trypsin digestion as described under "Materials and Methods." Soluble peptides were separated by pH 6.5 electrophoresis and descending chromatography. p (Pyrone F), o (Orange G), and v (valine) indicate the position of different markers after electrophoresis. xG is the position of the dye Xylene Cyanol FF after chromatography. Soluble tryptic peptides (T\(_1\)–T\(_{12}\)) and the material insoluble in pH 6.5 buffer (T\(_a\)) were characterized by amino acid composition. The amino-terminal residue of each peptide is underlined. T\(_i\), Gly, Lys; T\(_G\), Gln, Lys; T\(_S\), Met, Ile, Lys; T\(_T\), Thr, Gly, Leu, Tyr, Lys; T\(_S\), Asp, Thr, (Gly)s, Ala, Leu, Tyr, Phe, (Lys)s; T\(_{AS}\), Thr, Ser, (Glu)s, Gln, Pro, Ala, (Met)s, (Phe), (His)s, Lys; T\(_S\), Glu, Val, Met, Leu, Lys; T\(_S\), Asp, Gly, Ala, Phe, Lys; T\(_G\), (Asp)s, (Glu)s, Pro, Gly, Ala, Val, Met, Ile, Leu, Phe, (Lys)s; T\(_{AS}\), (Asp)s, (Glu)s, Pro, Gly, Ala, Val, Leu, Phe, Lys; T\(_T\), Asp, Gly, Leu, Arg, T\(_G\), Cys, (Asp), (Glu), Gly, Gly, Leu, Lys, Arg, T\(_T\), Cys, Asp, Ser, Pro, Ala, Val, Ile, Leu, Tyr, (Phe)s, His. There are only 2 arginine-containing peptides, i.e., T\(_{11}\) and T\(_{12}\). These have been characterized by amino acid sequence analysis as Glu-Asp-Leu-Arg (T\(_{11}\)) and Asp-Leu-Asp-Gln-Cys-Arg-Gly-Asp-Gly-Lys (T\(_{12}\)). Since the 10-kDa subunit contains 2.5% arginine (Table I), the presence of 2 unique arginine peptides argues for a polypeptide of about 85 residues, corresponding to a molecular weight of about 9,500. Note also that T\(_a\) is the amino-terminal tryptic peptide because the whole 10-kDa subunit also shows an amino-terminal proline.

T\(_{11}\) (Glu-Asp-Leu-Arg) and T\(_{12}\) (Asp-Leu-Asp-Gln-Cys-Arg-Gly-Lys). Given the amino acid composition of the small subunit, the presence of 2 arginine residues indicates a molecular weight of around 85 residues, i.e., 9,500. This conclusion is strengthened by the presence of only 2 cysteine-containing peptides, i.e., T\(_{12}\) and T\(_T\). Although the polypeptide molecular weight of the small subunit is about 10,000, the renatured protein behaves as a dimer in gel filtration experiments (Fig. 3A). This indication was confirmed by chemical cross-linking experiments with dimethyl suberimidate. Increasing concentrations of the reagent led to a decrease of the monomer species and a corresponding increased formation of a dimer band after analysis in SDS gels (Fig. 3).

In order to assess the quality of the renaturation process, the separated subunits, freed of urea, were mixed at equal molar concentrations in the absence of calcium. After incubation at room temperature for 30 min, the mixture was analyzed by gel filtration. Both subunits coeluted at a position corresponding to that of native protein I (Stokes radius of 41 Å) (Fig. 3A). Given the molecular weight of 85,000–90,000 determined by sedimentation equilibration (13), we conclude that protein I contains two copies each of the 36- and 10-kDa subunit. Whereas the 10-kDa subunit exists as a dimer under native conditions, the 36-kDa subunit can only form a dimeric structure when the 10-kDa dimer is also present.

**F-Actin- and Fodrin-Binding Properties of the Protein I Subunits—**The separated and renatured subunits were assayed for their ability to bind F-actin and nonerythroid spectrin as previously shown for the intact protein I (13). In buffers containing 100 mM KCl, 1 mM CaCl\(_2\), 2 mM MgCl\(_2\), 0.5 mM DTT, 1 mM NaN\(_3\), 20 mM imidazole-HCl, pH 7.5, binding of the 36-kDa subunit to F-actin was significantly reduced, as compared to intact protein I, while the 10-kDa subunit did not bind F-actin at all (Fig. 5). At a molar ratio of one 36-kDa polypeptide to five actins, the binding of the 36-kDa subunit was determined in several experiments using different preparations of 36 kDa. The average value of F-
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actin-bound 36 kDa was 35% upon low speed and 42% upon high speed centrifugation assays. These numbers are appreciably lower than the 94 and 95% found when the same assays were performed with intact protein I (Table II). When protein I was reconstituted from its isolated subunits, the F-actin binding activity reached the same high values documented previously for native protein I (Fig. 5 and Table II). SDS-gel electrophoresis showed that the harvested complexes contained as expected both the 36-kDa and the 10-kDa polypeptide. These were, however, thinner than those observed with either native or renatured protein I used at the same total concentration of the 36-kDa polypeptide (not shown). As previously demonstrated for intact protein I (13), the interaction of F-actin with the isolated 36-kDa subunit and the renatured protein I always required \(\text{Ca}^{2+}\) in concentrations above 50 \(\mu\text{M}\) and was consequently suppressed in EGTA-containing buffers (Fig. 5).

The interaction of the isolated and renatured subunits with pig brain fodrin was studied by gel filtration on Sepharose 4B as described before with protein I (13). Coelution of the 36-kDa subunit and fodrin indicative of a direct interaction was reduced to approximately 30% of the value obtained with native or renatured protein I (Fig. 6). On the other hand, fodrin and the purified 10-kDa subunit were clearly separated without any indication of interaction when the 36-kDa polypeptide was absent (Fig. 6). As seen previously with protein I, binding of the 36-kDa subunit required \(\text{Ca}^{2+}\) in concentrations above 50 \(\mu\text{M}\) (data not shown). We conclude from these results that the \(\text{Ca}^{2+}\)-dependent interaction of protein I with F-actin and fodrin is retained in the native monomeric 36-kDa subunit, although the extent of binding is noticeably reduced.

Calcium-induced Conformational Change of Protein I and its 36-kDa Subunit—UV difference spectroscopy of protein I revealed a strong calcium-dependent change in the environment of aromatic amino acids (Fig. 7A). Exposure to \(\text{Ca}^{2+}\) led to a decrease in absorbance in the range between 270 and 295 nm. According to Donavan (28) such a change usually indicates an increased exposure of the aromatic amino acids tyrosine and tryptophan to the aqueous solvent in the presence of the ligand. A similar \(\text{Ca}^{2+}\)-induced UV difference spectrum was observed with the isolated 36-kDa subunit (Fig. 7B), while the 10-kDa subunit failed to exhibit any change (not shown). The characteristic negative spectral differences were used to monitor \(\text{Ca}^{2+}\) binding in order to evaluate calcium binding constants of protein I and its 36-kDa subunit. In both cases, titration of the Ca-binding site(s), as shown in Fig. 7C for intact protein I, revealed an apparent binding constant of approximately \(10^4 \text{ M}^{-1}\).

A potential \(\text{Ca}^{2+}\)-induced conformational change in the polypeptide backbone was also explored by means of circular dichroism studies. The mean residue ellipticity in the absence of \(\text{Ca}^{2+}\) was \([\theta]_{522} = 15,300 \pm 300 \text{ (deg cm}^2/\text{dmol)}\) for protein I, 14,900 \(\pm 300\) (degrees cm\(^2\)/dmol) for the 36-kDa subunit, and 12,700 \(\pm 300\) (degrees cm\(^2\)/dmol) for the 10-kDa subunit (not shown). Addition of 1 mM \(\text{CaCl}_2\) slightly reduced the \([\theta]_{522}\) to 14,500 \(\pm 300\) (degrees cm\(^2\)/dmol) for intact protein I and 14,200 \(\pm 300\) (degrees cm\(^2\)/dmol) for the renatured 36-kDa subunit. No change of \([\theta]_{522}\) was observable for the 10-kDa subunit. Using the approximation of Chen et al. (29), the apparent \(\alpha\)-helix content of protein I, the 36-, and the 10-kDa subunit was estimated to be around 43, 42, and 34%, respectively. The decrease of \(\alpha\)-helix in protein I and the 36-kDa subunit due to the addition of \(\text{Ca}^{2+}\) is rather small, i.e. 1 to 2%.

The effect of \(\text{Ca}^{2+}\) on the UV absorption appeared to reflect a strong conformational change in the environment of tyro-

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**Fig. 6. Ca\(^{2+}\)-dependent interaction of renatured protein I and its subunits with fodrin.** Pig brain fodrin (1 mg/ml) was mixed with different protein I (1 mg/ml), 36-kDa (1 mg/ml) or 10-kDa (0.7 mg/ml) samples and applied to gel filtration on Sepharose 4B in 100 mM KCl, 0.5 mM DTT, 1 mM NaNO\(_3\), 1 mM \(\text{CaCl}_2\), and 10 mM imidazole-HCl, pH 7.4. Fractions were collected and monitored for absorbance at 280 nm. Aliquots of the protein peaks were analyzed by SDS-polyacrylamide gel electrophoresis (7.5-15% acrylamide) to determine complex formation. Polypeptides migrating with the fodrin peak in the different experiments are shown in lanes A, C, E, and G, whereas the protein composition of the protein I, 36-, 10-kDa, or reconstituted protein I peak is given in lanes B, D, F, and H, respectively. Experiments were performed with intact protein I (lanes A and B), renatured 36-kDa polypeptide (lanes C and D), renatured 10-kDa polypeptide (lanes E and F), or reconstituted protein I (lanes G and H). Reconstitution of protein I was obtained by mixing the renatured 36- and 10-kDa subunits prior to the fodrin-binding studies. Note that the interaction of the 36-kDa subunit with fodrin indicated by co-migration (lane C) is strongly reduced, as compared to intact (lane A) or reconstituted (lane G) protein I. The isolated 10-kDa subunit does not bind to fodrin (lane E).

**Fig. 7. Calcium-induced difference spectra of protein I and the 36-kDa subunit.** UV difference spectra of protein I (A) as compared to the base-line from 250 to 330 nm and of the renatured 36-kDa subunit (B) from 270 to 310 nm. The reference cell contained 0.1 mM EGTA whereas 1 mM \(\text{CaCl}_2\) was present in the sample cell. Difference spectroscopy of protein I was also used in \(\text{Ca}^{2+}\) titration experiments (C). Increasing effects are indicated by increasing \(\text{Ca}^{2+}\) concentration (0.06 mM, 0.2 mM, 0.5 mM free \(\text{Ca}^{2+}\)). Protein concentrations in the experiments shown were A, 0.93 mg/ml; B, 0.46 mg/ml; and C, 0.70 mg/ml. ABS, absorbance.
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**Fig. 8. Effect of Ca\(^{2+}\) on the fluorescence emission spectra of protein I and its subunits.** Protein I (0.05 mg/ml), 36 kDa (0.05 mg/ml), and 10 kDa (0.09 mg/ml) in 80 mM NaCl, 0.1 mM DTT, 50 mM Tris-HCl, pH 7.5, containing 0.2 mM EGTA (— — ) or 1 mM CaCl\(_2\) (— — ) were excited at 280 nm. The fluorescence emission spectra from 285 to 400 nm are shown for protein I (A), the 36-kDa (B), and 10-kDa polypeptide (C). The 10-kDa subunit (C) does not exhibit Ca\(^{2+}\)-induced changes in fluorescence emission.

To strengthen this hypothesis, we recorded the fluorescence emission spectra of protein I and its subunits. As seen in Fig. 8, protein I and the 36-kDa subunit showed very similar fluorescence emission when excited at 280 nm. The two emission spectra revealed almost identical features (not shown). The 10-kDa subunit, however, did not show Ca\(^{2+}\)-induced changes in fluorescence emission. The emission maximum appeared at 308 nm when an excitation wavelength of 280 nm was used (Fig. 8C). Excitation at 297 nm did not cause any fluorescence, in line with the lack of tryptophan in the 10-kDa subunit (Table I).

**Relationship of Protein I to a 35-kDa Protein from A-431 Cells**—After we completed the characterization of protein I and its subunits, Fava and Cohen (20) described the purification of a 35-kDa protein from A-431 human epidermoid carcinoma cells, which serves as a substrate for the EGF receptor tyrosine kinase. Although the isolation procedure of this protein (20) resembled that of protein I (13), i.e. extraction in EGTA-containing buffers, gel filtration, DEAE-, and CM-chromatography, some differences seemed obvious. Thus, the A-431 35-kDa protein behaved as a monomer in gel filtration and was not precipitated with an anti-34-kDa antiserum, raised against a protein of the 34-39-kDa family from chicken fibroblasts which serves as a substrate for the sarcoma virus-encoded tyrosine kinase (4). In order to compare protein I which exhibits immunological relationship to the fibroblast 34-39-kDa protein (13) with the 35-kDa protein from A-431 cells, we performed several experiments. First, we applied the A-431 35-kDa purification scheme of Fava and Cohen (20) to porcine intestinal epithelial cells and obtained homogeneous protein I exhibiting both the 36- and 10-kDa subunits. Second, we prepared an EGTA extract from A-431 cells according to Fava and Cohen (20). Immunoblotting of this extract after gel electrophoresis revealed a 35- and a 10-kDa polypeptide cross-reacting with protein I antiserum (Fig. 9). Protein I antibodies precipitated the 35- and 10-kDa polypeptides from the A-431 EGTA extract, leaving no major 35-kDa band in the supernatant when subsequently examined by SDS-polyacrylamide gel electrophoresis (data not shown). When A-431 cells were grown in \(^{32}P\)-containing medium in the presence of EGF, the immunoprecipitated 35-kDa polypeptide retained \(^{32}P\) label, and this was resistant to the treatment with base that hydrolyses phosphoserine and phosphothreonine esters and thus selects for phosphotyrosine (5). No phosphotyrosine-containing polypeptides in the 34-39,000-dalton mass range were detected in the supernatant after immunoprecipitation with protein I antibodies (data not shown). Third, the EGTA extract from A-431 cells was also subjected to gel filtration on Sephadex G-100. As described by Fava and Cohen (20), a major polypeptide migrating with an apparent molecular weight of about 35,000 on SDS-polyacrylamide gels was found after molecular sieving in fractions corresponding to a 35-kDa monomer. In addition, however, the same polypeptide was also found in the dimer position. Immunoblotting with protein I antibodies exhibited staining of the 35-kDa band in both fractions, indicating that this polypeptide exists in a monomeric as well as a dimeric form (Fig. 9). The protein I
antibodies also decorated a 10-kDa polypeptide which was present only in the dimeric 35-kDa eluted from the G-100 column (Fig. 9).

The combined results suggest that A-431 cells contain a 35-kDa protein immunologically related to the 36-kDa subunit of protein I. Whereas the majority of this protein (>60%) seems to exist as monomer in A-431 cells, there is also some dimeric species which contains the 10-kDa subunit as does protein I. In contrast, in intestinal epithelial cells more than 90% of the 36-kDa subunit is recovered in the dimer and less than 10% in the monomer position. Thus, in spite of their extensive similarities (see "Discussion"), the two proteins are not identical.

**DISCUSSION**

Protein I is a major protein of the intestinal epithelium (13) and resembles in its immunological and many biochemical properties the 36-kDa protein which serves as a substrate for the Rous sarcoma virus-encoded tyrosine-specific protein kinase (2-4, 7-9, 12). Although this substrate can be isolated from fibroblasts (4, 12), only the large amount of protein I available from porcine intestine allows detailed biochemical studies. Protein I of enterocytes is a tetrameric molecule containing two copies each of a 36-kDa and a 10-kDa subunit. It shows Ca$^{2+}$-dependent binding to F-actin and nonepithelial spectrin in vitro (13). The physiological importance of this in vitro interaction is not understood since it occurs only at Ca$^{2+}$ concentrations exceeding 50 μM, whereas the microfilament organization of the enterocyte brush border is thought to be particularly sensitive to elevated Ca$^{2+}$ levels (for review see Ref. 30). Nevertheless, as discussed below, these Ca$^{2+}$ concentrations induce a pronounced conformational change in protein I and its 36-kDa subunit. They are also necessary for the in vitro phosphorylation of a 35-kDa protein of A-431 cells by the EGF receptor kinase. As described below, this protein is immunologically and biochemically related to the 36-kDa subunit of protein I.

Here we have used denaturation/renaturation experiments to characterize the biochemical properties of the two subunits. Gel filtration in the presence of 9 M urea resulted in the separation of the 36-kDa and the 10-kDa polypeptide. Dialysis against urea-free buffers led to renatured subunits. Gel filtration in the presence of 9 M urea resulted in the renatured 36-kDa subunit alone is a monomer while the renatured protein I and its 36-kDa subunit. They are also necessary for the in vitro phosphorylation of a 35-kDa protein of A-431 cells by the EGF receptor kinase. As described below, this protein is immunologically and biochemically related to the 36-kDa subunit of protein I.

The Ca$^{2+}$-dependent F-actin and fodrin-binding properties of protein I are already displayed by the isolated 36-kDa subunit, although dimerization of the 36-kDa polypeptide by adding the 10-kDa dimer enhances the binding considerably. The monomeric 36-kDa subunit not only binds but also bundles F-actin filaments. Thus, it seems that the 36-kDa subunit contains at least two actin-binding sites as a presupposition for bundling. Several other basic proteins are reported to cross-link actin filaments and induce gelation (32). Although this situation differs from bundle formation, proteins such as aldolase or lysozyme also need to have two or more actin-binding sites when their cross-linking ability is considered. In the case of the 36-kDa subunit, the total number as well as the diameter of the actin-36-kDa bundles increases markedly upon dimerization. If the 36-kDa subunit itself already contains two actin-binding sites, it seems possible that the F-actin-36-kDa equilibrium shifts towards complex formation when dimerization of the 36-kDa subunit is induced in the presence of the 10-kDa dimer.

The physicochemical properties of protein I and its subunits con firm our previous view as to the presence of a Ca$^{2+}$-binding site (13). We have located such a site to the 36-kDa subunit. Ca$^{2+}$ binding of the 36-kDa subunit, intact, and reconstituted protein I results in a pronounced conformational change characterized by an altered environment of some of the aromatic amino acid residues. UV difference and fluorescence spectra indicate that tyrosine and tryptophan residues become exposed to a more aqueous environment in the presence of Ca$^{2+}$. Titration experiments show that the Ca$^{2+}$-induced conformational change coincides with the Ca$^{2+}$-induced acquisition of binding to F-actin or fodrin. All three properties become first noticeable at a free Ca$^{2+}$ concentration of 50 μM when Mg$^{2+}$ is present at 2 mM. Our results do not allow a conclusion as to the total number of such Ca$^{2+}$-binding sites or to the absence of additional sites not resulting in a conformational change seen by difference spectroscopy. Currently, it is premature to speculate whether one of the tyrosines of the 36-kDa subunit, which is involved in the Ca$^{2+}$-dependent conformational change, is the target of the viral tyrosine kinase. However, future amino acid sequence data may be able to assess this possibility since the sole tryptophan residue of the 36-kDa subunit also seems affected by the environmental change. Our own in vitro phosphorylation studies of protein I have been hampered by the poor stoichiometry of phosphate incorporation. Although a tyrosine-specific kinase from Fujinami sarcoma virus-transformed...
cells (33, 34) is able to phosphorylate protein I, the degree of in vitro phosphorylation is rather low.2 Whereas autophosphorylation of the Fujinami virus kinase and phosphorylation of substrates such as casein were markedly reduced in the presence of 500 μM Ca2+, the phosphorylation of protein I was not inhibited but indeed slightly enhanced by calcium.2 This observation indicates that at least in vitro phosphorylation of protein I by the viral kinase may be enhanced by Ca2+.

Recently, Fava and Cohen (20) reported that the in vitro phosphorylation of a 35-kDa polypeptide of A-431 human epidermoid carcinoma cells by the EGF receptor kinase is regulated by Ca2+. Tyrosine phosphorylation was greatly enhanced at Ca2+ concentrations exceeding 25 μM and could under optimal conditions approach 15%. Since this high Ca2+ requirement approximately coincides with the high Ca2+ levels necessary to induce a conformational change in protein I and its 36-kDa subunit, we have performed a preliminary characterization of the 35-kDa acceptor protein of A-431 cells. This protein as well as protein I show a very similar fractionation and purification scheme, including the specific solubilization from cytoskeletal and membrane fractions by EGTA-containing buffers. In addition, the EGTA extract of A-431 cells contains a 35- and a 10-kDa polypeptide cross-reacting with protein I antibodies. By gel filtration, the 35-kDa protein from A-431 cells immunologically related to protein I was found to elute both as a monomer and a dimer. Whereas the monomer fractions are free of a cross-reacting 10-kDa polypeptide, the dimer fractions reveal the immunologically related 10-kDa species. Thus, as in protein I, the A-431 protein can form a dimer in the presence of a 10-kDa polypeptide. Interestingly, Fava and Cohen (20) monitoring their purification of the 35-kDa polypeptide by Ca2+-dependent phosphorylation using a crude EGF receptor kinase found acceptor activity only in the monomer position. The combined data open the possibility that the 35-kDa dimer from A-431 cells is only poorly phosphorylatable in vitro by the EGF receptor kinase. In contrast, the monomeric 35-kDa polypeptide of A-431 cells, which very likely resembles the large protein I subunit, seems a much better substrate. This suggestion is supported by our preliminary experiments on in vitro phosphorylation of protein I and its 36-kDa subunit by membrane vesicles of A-431 cells, which contain EGF receptor kinase (data not shown). It remains, however, unclear why different amounts of the 35- to 36-kDa polypeptides exist as dimers in different cell types. Thus, we have found more than 90% dimer in intestinal cells but only up to 40% dimer in A-431 cells. One possibility to account for this difference may lie in slightly different primary structures of the subunits in different cell types. In this respect, we note a reproducible difference in apparent molecular weight of the large subunit, i.e. 36,000 in enterocytes and 35,000 in A-431 cells.

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2 V. Gerke, P. Donner, K. Moelling, and K. Weber, unpublished results.