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Biochemistry

A human protein related to the major envelope protein of murine mammary tumor virus: Identification and characterization

(human milk protein/viral glycoprotein)

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ABSTRACT **A human milk protein was isolated to apparent homogeneity and shown to be immunologically related to gp55, the major envelope glycoprotein of murine mammary tumor virus. Through the development of ultrasensitive protocols, which have wide applicability, immunological relatedness was corroborated by the demonstration of homologous protein sequences between the human and viral proteins.**

Several lines of evidence have suggested the association of virus-related RNA (1, 2) and proteins (3, 4) with human mammary tumors (5, 6). Most importantly, breast cancer patients can mount a specific cell-mediated immune response to gp55 (7), the 55,000-dalton glycoprotein of murine mammary tumor virus (MuMTV), as monitored by the leukocyte migration inhibition test, which correlates with a favorable prognosis (8). Similarly, virus-like particles (9) and RNA-directed DNA polymerase (10) have been reported in human milk.

MuMTV-related human proteins have been indirectly ascertained by monitoring humoral or cell-mediated immune responses against MuMTV or by immunohistochemical assays (3, 11) of human mammary tissue sections and immunofluorescence staining (4) of an established cell line (MCF-7), derived from a pleural effusion of a breast cancer patient, with monospecific antisera directed against gp55. A direct demonstration of sequence homology between a human protein and a related viral component (gp55) would corroborate and extend these cited observations. This approach forms the basis of the data presented here.

MATERIALS AND METHODS

Purification of Viral Glycoproteins. The major envelope glycoproteins [gp55(RIII) and gp50(A)] of MuMTV(RIII) and MuMTV(A), respectively, were purified by ion-exchange and molecular sieving chromatography after detergent disruption of isopycnically banded milk-borne virus (12) derived from isogeneic RIII and A mouse strains, respectively.

Preparation of Human Milk Particulate Fractions. Aliquots (12–15 ml) of individual human milks from approximately 300 separate donors were thawed and clarified as described (13), and the skim-milk fraction was layered onto a 5-ml layer of 20% glycerol in buffer A (10 mM Tris·HCl, pH 8.3/0.15 M NaCl/2 mM EDTA). Unless otherwise stated, all procedures were carried out at 4°C. After centrifugation at 25,000 rpm (SW 25.1 rotor) for 60 min, the supernatant was removed by careful suction; each pellet was resuspended in an equal volume of buffer A and glycerol and stored at −20°C until used.

Ion-Exchange Chromatography of Solubilized Human Milk Particulate Fractions. Prior to chromatography, an aliquot of the stored pool, equivalent to approximately 1 liter of whole human milk, was diluted with buffer A and centrifuged at 25,000 rpm (SW 27 rotor) for 60 min. The supernatant was discarded, and the pellet was resuspended in 30 ml of solubilization buffer [0.2 M sodium phosphate, pH 6.8/10 mM EDTA/0.2% 2-mercaptoethanol/1.0% (vol/vol) Nonidet P-40] (12). After overnight solubilization with gentle stirring at 4°C, insoluble debris was removed by centrifugation at 10,000 rpm (SS-34 rotor). The clarified supernatant was diluted 1:10 with DEAE-cellulose buffer (20 mM Tris·HCl, pH 7.2/0.2% 2-mercaptoethanol/0.2% Nonidet P-40/30% glycerol) and applied to a DEAE-cellulose column (1.4 × 26 cm) equilibrated with the same buffer at a flow rate of 50 ml/hr. The column was washed with two column volumes of the same buffer and eluted with DEAE-cellulose buffer containing 0.4 M KCl. The fractionation was continuously monitored at 280 nm. Fractions were pooled (fractions 2–6, Fig. 1*A*) and concentrated by means of dialysis under reduced pressure.

Micro-Ouchterlony Assays. Immunodiffusion assays were performed by the technique of Charney *et al.* (14), modified by the use of Coomassie blue R250 (Colab Laboratories, Glenwood, IL), 0.25% in glacial acetic acid/water/methanol, 11:4:5 (vol/vol), for staining.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Disc-gel polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed as described (15). Slab-gel electrophoresis proceeded by the technique of Laemmli (16). The resolving gel consisted of 10% acrylamide (105 × 105 × 1.5 mm) with a 3% stacking gel. Disc and slab gels were stained with Coomassie blue or periodic acid-Schiff reagent as described (15).

***In Situ* Detection of Antibody–Antigen Complexes After Gel Electrophoresis.** Subsequent to slab-gel electrophoresis according to Laemmli (16), $NaDodSO_4$ was removed by immersing the gel overnight in methanol/glacial acetic acid/water, 50:7.5:42.5 (vol/vol), followed by rehydration in phosphate-buffered saline (pH 7.4) at room temperature with multiple buffer changes (every 30 min) until the solution was at neutral pH. Antigen–antibody complexes were detected *in situ* by the technique of Olden and Yamada (17) with modifications. The slab gel was bathed overnight at 37°C in a 1:10 dilution of preimmune or monospecific anti-gp55 (α-gp55) goat serum IgG; uncomplexed antibody was removed from the gel by several washes in phosphate-buffered saline during a 48- to 72-hr period and incubated in the presence of rabbit anti-goat IgG (Miles-Yeda, Rehovot, Israel) at 37°C for 90 min. Excess rabbit serum was removed by several washes in phosphate-buffered saline (48–72 hr) and the gels were stained with Coomassie brilliant blue (15). Densitometer gel scans (570 nm)

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Abbreviations: MuMTV, murine mammary tumor virus; gp55(RIII), 55,000-dalton glycoprotein of MuMTV(RIII); gp50(A), 50,000-dalton glycoprotein of MuMTV(A); α-gp55, monospecific goat antiserum against gp55.

were performed with a Gilford 2400S spectrophotometer equipped with an automatic gel scanning apparatus.

Amino-Terminal Residue Assays. Proteins were eluted from gels after direct staining. The Coomassie blue stain was removed by maceration of the gel section in methanol/glacial acetic acid/water, 50:7.5:42.5, and the gel fragments were recovered by low-speed centrifugation and lyophilized. Proteins were eluted from the gel fragments by overnight incubation at 37°C in 100 μl of 5 mM $NaHCO_3$/0.05% $NaDodSO_4$ followed by low-speed centrifugation to remove the gel fragments (18). The supernates were recovered, lyophilized to dryness, and resuspended in 20 μl of 0.2 M $NaHCO_3$ (pH 9.8). Dansylation was achieved by addiion of 20 μl of [^{14}C]dansyl chloride (1 mg/ml; 62.2 Ci/mol; New England Nuclear) and incubation at 37°C for 45 min (1 Ci = 3.7×10^{10} becquerels). The reaction was terminated by the dropwise addition of 50 μl of 20% trichloroacetic acid. The labeled proteins were recovered by centrifugation, washed twice with 200 μl of 1 M HCl to remove unreacted reagent and byproducts, and taken to dryness. Labeled NH_2 termini were released by acid hydrolysis in 50 μl of 6 M HCl for 5 hr at 105°C and the hydrolysate was dried under reduced pressure in the presence of H_2SO_4 and NaOH pellets. NH_2 termini were determined by resuspension of the dried hydrolysates in 5–10 μl of 50% aqueous pyridine and spotting onto 5 × 5 cm polyamide sheets (Cheng Chin; Gallard-Schlesinger, Carle Place, NY); a standard, unlabeled dansylated amino acid mixture (5.2 ng each) was treated the same way. Two-dimensional thin-layer chromatography was performed according to Bertrand *et al.* (19), followed by radioautographic detection of the NH_2 termini by affixing the dried chromatograms to a glass plate which was directly apposed to Kodak NS-5T x-ray film. NH_2 termini were determined by coincidence of radiolabeled amino acids to fluorescent standards on the same plate.

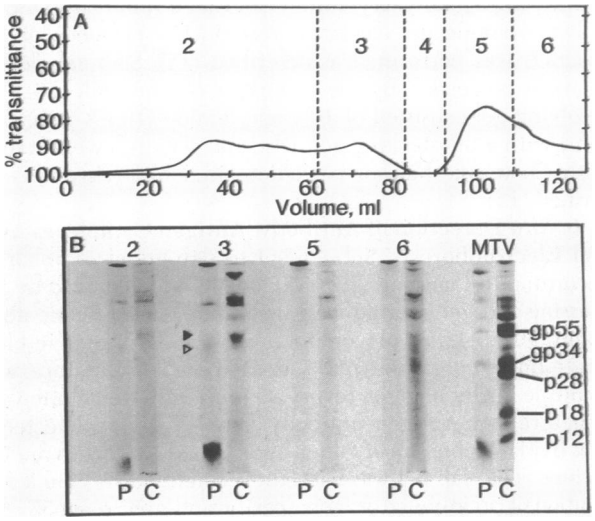

FIG. 1. Absorbance profile (280 nm) (*A*) and $NaDodSO_4$/polyacrylamide gel electrophoresis (*B*) of a detergent-solubilized particulate fraction prepared from human milk after ion-exchange chromatography on DEAE-cellulose. For $NaDodSO_4$/polyacrylamide gel analyses, 30 μg of protein from each fraction (2–6 in *A*), determined by the Lowry procedure (20) as modified by Wang and Smith (21) with bovine serum albumin as the standard, was electrophoresed on disc gels which were sectioned lengthwise and stained with periodic acid-Schiff reagent (P) and Coomassie blue (C) as described (15). For fraction 3 in *B*, the densely staining C band corresponds to a lightly staining P band (closed arrowhead); the lower-molecular-weight, more densely staining P band (open arrowhead) is associated with a minor C band.

Tryptic Peptide Mapping Analyses. After slab-gel electrophoresis, direct staining with Coomassie blue, and destaining of the protein bands with methanol/acetic acid, as described above, the gel fragments were recovered by low-speed centrifugation and lyophilized after the supernates were discarded. Trypsin digestion was performed by rehydration of the gel fragments in 300 μl of 50 mM triethylamine-HCl buffer (pH 8.8) containing 5.75 mM $CaCl_2$. TPCK-trypsin [L-(tosylamido-2-phenyl)ethylchloromethyl ketone-treated trypsin; Worthington] was then added at a concentration equivalent to approximately 2% of the protein to be digested. After overnight digestion at 37°C, gel fragments were removed by low-speed centrifugation and the supernates were lyophilized to dryness. Tryptic peptides were then resuspended in 200 μl of 0.2 M $NaHCO_3$ and treated with [^{14}C]dansyl chloride as described above for amino-terminal assays. The samples were dried in a stream of N_2 and resuspended in 20 μl of 95% ethanol. For two-dimensional analyses, samples were spotted on polyamide sheets (7.5 × 7.5 cm) and developed twice in 7.5% formic acid, with drying between the first and second solvent runs. Chromatography in the second dimension was performed in benzene/glacial acetic acid, 3:1 (vol/vol). After drying in a cool air stream, the chromatograms were directly apposed to Kodak NS-5T x-ray film for radioautographic detection of labeled peptides.

RESULTS

Partial Purification of MuMTV-Related Protein from Human Milk. Detergent disruption of the human milk particulate pool, followed by ion-exchange chromatography, resulted in the absorbance profile illustrated in Fig. 1*A*. Of the 20 mg of protein applied to the column (fraction 1), approximately 70% was recovered in the effluent fractions (fractions 2–6). Unadsorbed protein appearing in the application and wash pool (fraction 2) constituted 13.5% of the total protein recovered, whereas fractions 3, 5, and 6, eluted with 0.4 M KCl, contained 16.9, 53.8, and 15.8%, respectively. The results of polyacrylamide gel electrophoresis of $NaDodSO_4$-denatured samples are shown in Fig. 1*B*. Only fraction 3 contained a protein band with a size of approximately 55,000 daltons that stained lightly with periodic acid-Schiff reagent. The significance of this observation became apparent from additional assays described below; i.e., fraction 3 contained a glycoprotein

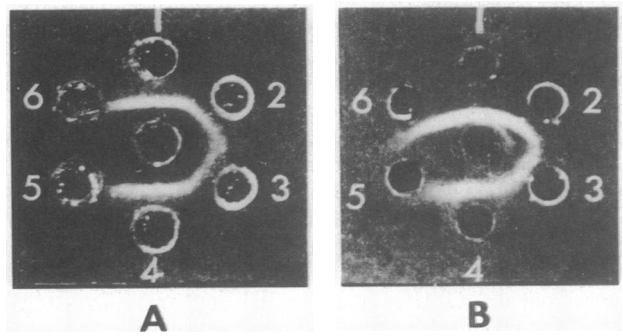

FIG. 2. Micro-Ouchterlony immunodiffusion assays demonstrating immunological crossrelatedness between a protein component of human milk (fraction 3, Fig. 1*A*) and the major glycoprotein of MuMTV (gp55). Rabbit antiserum (central wells) against disrupted MuMTV(RIII) was prepared and absorbed against virus-free CBL milk as described (14). (*A*) Wells 1 and 4, gp55 released from MuMTV (RIII) disrupted with 5% diethyl ether in phosphate-buffered saline; wells 2 and 3, human milk fraction 3 (Fig. 1*A*); wells 5 and 6, human milk fraction 2 (Fig. 1*A*). (*B*) Wells 1 and 4, gp55 purified from MuMTV (RIII) (12); wells 2 and 3, human milk fraction 3; wells 5 and 6, DEAE-cellulose buffer.

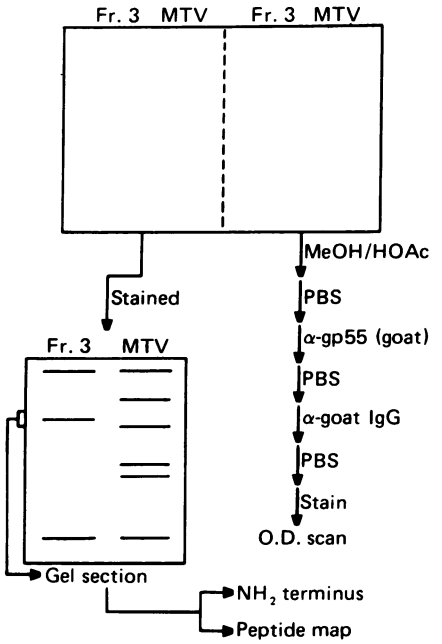

FIG. 3. Experimental protocols for the simultaneous demonstration of immunological relatedness and structural homologies between gp55 of MuMTV and a human milk protein in fraction 3 (Fig. 1*A*). After slab-gel electrophoresis, immunologically related proteins were demonstrated with goat antiserum prepared against gp55 (α-gp55) subsequent to the removal of $NaDodSO_4$ from the gel with methanol/acetic acid (MeOH/HOAc) and rehydration in phosphate-buffered saline (PBS) (17). Assays for structural homologies between related proteins, demonstrated as above, were done on protein bands that were stained directly after electrophoresis. The bands were destained by maceration in MeOH/HOAc and the proteins were characterized by determination of NH_2 termini (Fig. 5), tryptic peptide mapping (Fig. 6), and determination of NH_2-terminal residues in common peptides (Fig. 7).

related to the major envelope protein of MuMTV (gp50–gp55).

Of the fractions displayed in Fig. 1, only fraction 3 routinely contained a MuMTV-related protein. As shown in Fig. 2*A*, polyvalent rabbit antiserum against disrupted MuMTV of the RIII mouse strain [MuMTV(RIII)] produced an antigen–antibody precipitin line with human fraction 3 that was confluent with a protein from ether-disrupted MuMTV (RIII) containing mainly gp55. That the latter protein was gp55 is evident from assays presented in Fig. 2*B*: a confluent precipitin line extended between the response of antibody against the human fraction and against purified gp55(RIII). Additionally, these observations were corroborated by immunodiffusion assays utilizing monospecific goat antisera against gp50(A) or gp55(RIII) (not shown). Finally, no human fractions reacted with monospecific goat antisera prepared against p28 and p12, the major internal proteins of MuMTV (RIII). It thus seemed pertinent to simultaneously demonstrate immunological relatedness and the structural homologies to verify this phenomenon; this direct approach is detailed below.

Experimental Rationale. As shown in the diagram presented in Fig. 3, the size of the viral gp55-related human milk protein could be determined after slab-gel electrophoresis by *in situ* localization of antigen–antibody complexes by the protocol of Olden and Yamada (17). Furthermore, this technique permits several characterizations of the protein band localized by directly staining a slab gel that had been simultaneously electrophoresed but not treated with the antibody. These characterizations include the NH_2 terminus, tryptic peptide map, and compositional analyses of some of the peptides, which are discussed below.

Identification and Characterization of MuMTV-Related Human Milk Protein. According to the protocols diagrammed in Fig. 3, it was initially necessary to localize and identify which human milk protein in fraction 3 was related to gp55 after $NaDodSO_4$/polyacrylamide gel electrophoresis. By a modified technique of Olden and Yamada (17), the results presented in Fig. 4 were obtained. The enhanced staining associated with

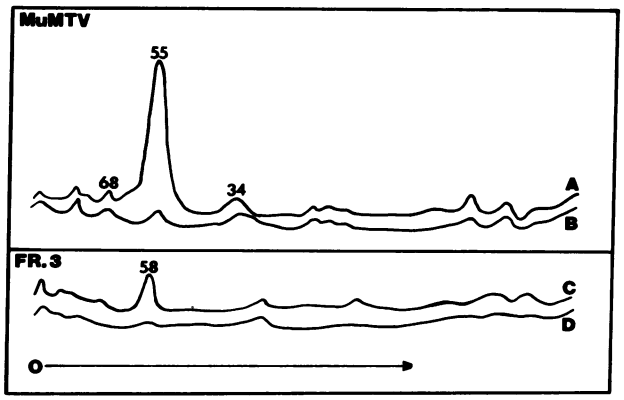

FIG. 4. *In situ* detection of gp55-related human milk protein by specific antigen–antibody interactions after slab gel electrophoresis. Approximately 30 μg of human milk fraction 3 (FR. 3) and MuMTV (RIII) was disrupted with $NaDodSO_4$ and subjected to slab-gel electrophoresis (16). $NaDodSO_4$ was removed by immersion in methanol/acetic acid, followed by localization with specific antisera. Curves A and B, MuMTV(RIII) treated with monospecific goat IgG (α-gp55) and preimmune goat IgG, respectively. Curves C and D, human milk fraction 3 (Fig. 1*A*) incubated in the presence of goat IgG (α-gp55) and preimmune goat IgG, respectively. Numerical designations indicate positions of gp68 (68), gp55 (55), gp34 (34), and the human milk protein (58).

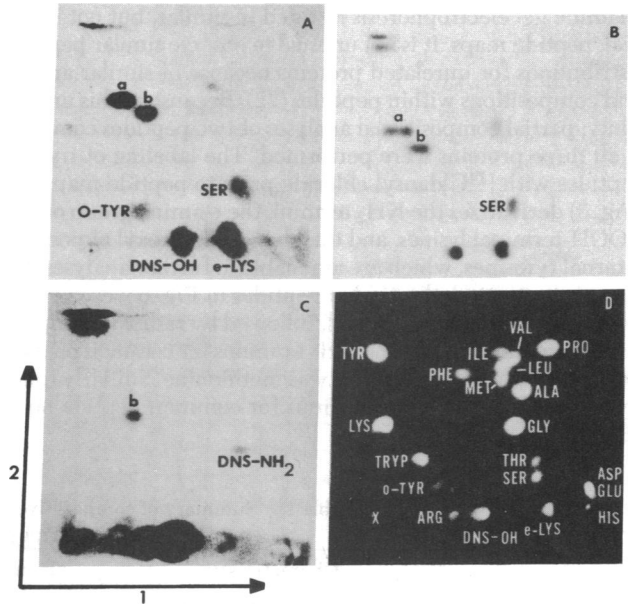

FIG. 5. Amino-terminal residue determinations by treatment of proteins with [^{14}C]dansyl chloride followed by acid hydrolysis, polyamide thin-layer chromatography, and radioautography. (*A* and *B*) Radioautographic detection of [^{14}C]dansylated serine as the NH_2 terminus of gp55 and the human milk protein, respectively; (*C*) reagent blank; (*D*) separation of fluorescent dansylated amino acid mixture by thin-layer chromatography. DNS-OH, dansylic acid; DNS-NH_2, dansyl amide; a and b, dansylated byproducts that do not coincide with any of the standard, fluorescent amino acids. ε-LYS and o-TYR, monodansylated lysine and tyrosine, respectively, substituted at the designated position.

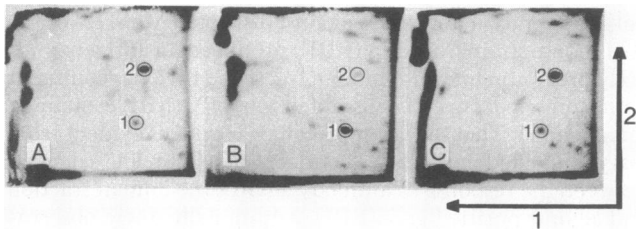

FIG. 6. Tryptic peptide mapping analyses of gp55 (*A*), the human milk protein gp58 (*B*), and gp50 (*C*), demonstrating common peptide sequences among these immunologically related proteins. Circled, common peptides (nos. 1 and 2) for all three proteins were further analyzed for *de novo* NH_2 termini (see Fig. 7).

the gp55 band of MuMTV is due to anti-gp55 (α-gp55) antibody–antigen complexes. The specificity of this complex formation is apparent from the lack of reactivity of α-gp55 with other MuMTV proteins and by an absence of complex formation with preimmune serum. In a similar fashion, a specific antigen–antibody complex was observed between a protein from human milk fraction 3, with an apparent molecular weight of 58,000 and α-gp55, corroborating the data previously obtained through immunodiffusion assays.

NH_2-terminal amino acid residues were determined for gp55(RIII) and the related human milk protein, gp58. As illustrated in Fig. 5, each of these proteins possessed a serine residue as the NH_2 terminus. The lack of additional NH_2 termini indicates, in addition, that each preparation is relatively homogeneous, which is necessary for meaningful comparative tryptic peptide mapping studies.

Immunological relatedness among proteins constitutes indirect evidence for shared amino acid sequence homologies that are amenable to corroboration by tryptic peptide mapping. As demonstrated in Fig. 6, trypsin digestions of gp50(A), gp55(RIII), and the human milk protein (gp58) after polyacrylamide gel electrophoresis resulted in similar, but not identical, peptide maps. It is not unusual to observe similar peptide distributions for unrelated proteins because of similar amino acid compositions within peptides (22). Because of this uncertainty, partial compositional analyses of two peptides common to all three proteins were performed. The labeling of tryptic peptides with [^{14}C]dansyl chloride prior to peptide mapping (Fig. 6) derivatizes the NH_2 termini, the ϵ-amino group of the COOH-terminal lysines, and the phenolic hydroxyl of possible internal tyrosines, which are amenable to further analyses. By way of illustration, the circled peptides in Fig. 6 were hydrolyzed and rechromatographed, followed by radioautography. Fig. 7 demonstrates that the NH_2 terminus for common peptide no. 2 of all three preparations was methionine. Smilarly, phenylalanine was the NH_2 terminus for common peptide no. 1 (Fig. 6).

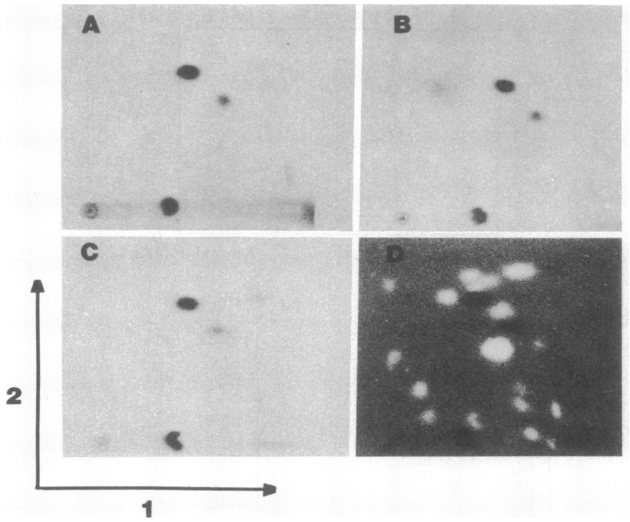

FIG. 7. Determination of *de novo* NH_2 termini for common peptide 2 (circled in Fig. 6). Peptides 1 and 2 (Fig. 6), localized by radioautographic detection, were scraped from the plate and eluted overnight with 200 μl of 95% ethanol. Polyamide was removed by centrifugation and the supernates were dried with a stream of N_2. Dansylated NH_2 termini were released by resuspension of the peptides in 50 μl of 6 M HCl and hydrolysis at 105°C. Finally, the hydrolysates were taken to dryness under reduced pressure, resuspended in 50% aqueous pyridine, and analyzed by the techniques given in the legend for Fig. 5. The NH_2 terminus for common tryptic peptide 2 (Fig. 6) was methionine for gp55 (*A*), the human milk protein gp58 (*B*), and gp50 (*C*). (*D*) Coincidence of radiolabeled methionine released from gp50 (*C*) with fluorescent, dansylated methionine from a standard amino acid mixture (see Fig. 5*C*) by overlaying the developed x-ray film on top of the chromatogram. In the same fashion, the NH_2 terminus for common peptide 1 (Fig. 6) for gp55(RIII), gp50(A), and the related human milk protein was determined to be phenylalanine (not shown).

DISCUSSION

The data summarized in Table 1, comparing the salient properties of gp50(A), gp55(RIII), and the human milk protein (gp58), provide conclusive evidence for structural homologies among these proteins that are reflected in immunological relatedness (Figs. 2 and 4). With regard to the immunodiffusion assays presented in Fig. 2, we did not observe "spurring," which could be interpreted as a "reaction of partial identity." Although these reactions are expected when related, but not identical, antigens are compared, they are not readily discernible through immunodiffusion assays of related antigens of MuMTVs (23). For example, in our own immunodiffusion studies, a similar line of identity, without spurring, was seen between gp50(A) and gp55(RIII) when they reacted against the same sera.

An aspect of the *in situ* detection technique (Fig. 4) should also be noted. The enhanced Coomassie blue staining caused

Table 1. Summary of comparative studies of human milk protein (gp58) and the major envelope glycoproteins of MuMTV

Characteristic	Human protein	gp55(RIII)	gp50(A)
Elution from DEAE-cellulose	0.4 M KCl	Application and wash	
Molecular weight	58,000	55,000	50,000
Periodic acid-Schiff reagent	Positive	Positive	Positive
NH_2 terminus	Ser	Ser	ND*
Tryptic peptide maps	Share common sequences, but nonidentity is also obvious		
NH_2 termini			
Peptide 1	Phe	Phe	Phe
Peptide 2	Met	Met	Met
Immunological assays	Share group-specific determinants		

* Not determined.

by specific antibody–antigen interactions is apparently the result of two phenomena: (*i*) protein concentration is increased because of specific immunologic interactions, and (*ii*) these complexes prevent elution of protein from the gel, which apparently occurs when complexes are not formed. These account for the paucity of bands observed as well as the decreased band intensities.

Comparative tryptic peptide mapping studies reveal structural homologies by virtue of compositional similarities. From the consideration that a specific amino acid will have a probability of occurrence within a protein sequence equivalent to the proportion of that residue to the total amino acid composition of a protein (24), it is highly improbable that the same NH_2 terminus for common peptides 1 and 2 of gp55(RIII), gp50(A), and the human milk protein could have been found by random probability alone. For example, the proportion of phenylalanine in gp55(RIII) and gp50(A) is 23/411 and 22/397, respectively (25); similarly, methionine occurs with a frequency of 3–4 residues for each of these proteins. Assuming a similar distribution of these residues in the human milk protein, chance alone could not account for the same NH_2 termini for peptides 1 and 2. It is even more unlikely when one considers that these *de novo* NH_2 termini were originally "nearest neighbors" of lysine or arginine residues.

Contamination of human milk with MuMTV was a primary concern from the initiation of these studies, and every effort was made to eliminate this possibility. In addition to our "isolating" the human milk investigation from animal virus work, various characteristics of the human protein distinguish it from the major group-specific antigen of MuMTV (gp50–55). As shown in Fig. 1, the major portion of the MuMTV-related human protein routinely elutes with 0.4 M salt (fraction 3) whereas gp55(RIII) and gp50(A) are not adsorbed under the same conditions and elute within the application and wash fraction (fraction 2) (12). In addition, the molecular weight and peptide map of the human protein, although similar to those of the related MuMTV proteins, differ to an extent sufficient to distinguish the human protein from the MuMTV proteins.

Taken *in toto*, the data presented provide strong evidence that human milk contains a protein that is related to the major envelope glycoprotein of MuMTV. Assay of individual milks should clarify whether the expression of this protein is correlated to a generalized lactogenic hormone response or whether expression is more restricted (i.e., in those women who have been assessed as high risk by various criteria). Approximately 18 of the 300 women (6%) from whom we derived our milk pool will eventually develop breast cancer. In addition, a small amount of milk from monolateral mastectomy patients was included. Finally, because of the virulytic capacity of human milk (26), it is probable that the "soluble" milk fraction may ultimately prove to be a more dilute, but better source, of the MuMTV-related protein.

The protocols developed here are useful in the simultaneous demonstration of immunological relatedness and structural homologies among proteins. Additionally, techniques presently exist with greater detection sensitivities in gels *in situ* than used in this study (17, 27). With regard to structural studies (tryptic peptide mapping and NH_2 termini), the practical lower limit from our studies was approximately 0.05–0.1 nmol of protein; however, the availability of [^{35}S]dansyl chloride of high specific activity would perhaps permit lower detection sensitivities.

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