# Bovine PAS-6/7 Binds $\alpha_V\beta_5$ Integrin and Anionic Phospholipids through Two Domains[†]

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ABSTRACT: Bovine milk fat globule membranes are a rich source of glycoproteins PAS-6 (52 kDa) and PAS-7 (47 kDa). They are glycosylation variants sharing a common polypeptide core. The PAS-6/7 protein consists of two EGF-like domains and a tandem repeated structure with a high degree of similarity to the C1 and C2 domains found in blood-clotting factors V and VIII. The second EGF-like domain contains an RGD cell adhesion sequence with the possibility of binding integrins, while the C-terminal end of the C2-like domain contains a probable amphipathic α-helix. Using a PAS-6/7 column, bovine $\alpha_V\beta_5$ integrin was purified from mammary gland tissue by affinity chromatography and characterized by Western blotting and N-terminal sequencing. The interaction between PAS-6/7 and the $\alpha_V\beta_5$ integrin was shown to be RGD dependent. Lipid binding assays showed that PAS-6/7 binds to surfaces of phosphatidylserine, -inositol, and -glycerol, and their precursor, phosphatidic acid, but not phosphatidylcholine. Furthermore, PAS-6/7 displayed the highest affinity toward a total lipid fraction derived from the milk fat globule membrane as compared to pure phospholipids. Using Western blotting technique, PAS-6/7 was shown to be widely expressed in a number of tissues. These results show that PAS-6/7 is a common protein which can bind to membranes by two distinct mechanisms, one through affinity to integrin $\alpha_V\beta_5$ and another by direct binding to phospholipids.

Glycoproteins PAS-6 and PAS-7 are two abundant peripheral proteins associated with the bovine milk fat globule membrane (MFGM).[1] PAS-6 and PAS-7 have been characterized to be glycosylation variants sharing a common protein core named PAS-6/7, and the full-length cDNA encoding PAS-6/7 has been obtained (Hvarregaard et al., 1996). Analysis of the amino acid sequence showed that PAS-6/7 has two N-terminal EGF domains, of which the second contains an RGD cell adhesion sequence motif. The RGD sequence in a number of proteins has been shown to be involved in the binding of integrins of which at least 20 are known. These integrins are $\alpha/\beta$ heterodimers, and they are involved in adhesion, differentiation, proliferation, and death of cells [reviewed by Hynes (1992)]. The RGD sequence and position are conserved in human BA46 and murine MFG-E8 (Couto et al., 1996; Stubbs et al., 1990), which are PAS-6/7 counterparts. Human BA46 is overexpressed in mammary gland tumors and promotes cell attachment in an RGD-dependent manner (Peterson et al., 1995). Integrins have been investigated in relation to breast cancer and tissue differentiation, and evidence is mounting on the role of these receptors in mammary gland differentiation [reviewed by Alford and Taylor-Papadimitriou (1996)].

The EGF domains of PAS-6/7 are followed by a tandem repeated domain with high similarity to the membrane associated C1 and C2 domains of blood-clotting factors V and VIII. Numerous studies report the C1−C2 domain of clotting factor VIII to be at least partly responsible for platelet membrane association during activation of blood-clotting factor X (Foster et al., 1990; Scandella et al., 1995). This observation was supported by structural analyses on a synthetic peptide consisting of the C-terminal part of the C2 domain of factor VIII. These analyses showed the presence of an amphipathic α-helix (Gilbert & Baleja, 1995). It is likely that PAS-6/7 also contains an amphipathic α-helix in the corresponding region, due to the conservation of functionally important amino acid residues (Hvarregaard et al., 1996). Furthermore, PAS-6/7 membrane association was evident from immunohistochemical experiments showing intensive staining on bovine epithelial cell membranes oriented toward alveolar lumina (Aoki et al., 1994). However, PAS-6/7 is also found to be loosely associated with MFGM as evident by a substantial release of the proteins during the process of MFGM isolation and by the ability to be extracted from MFGM with different salt solutions (Mather & Keenan, 1975; Kanno & Kim, 1990). Factor VIII interaction with the platelet membrane has been shown to be dependent on negatively charged phospholipids, with emphasis on phosphatidylserine (Bloom, 1987). These anionic phospholipids also are found in similar amounts in the MFGM (Jensen et al., 1991) and provide an explanation for the association of PAS-6/7 with the membrane.

The function of PAS-6/7 is not known, but it is distributed on a number of glandular epithelial cells and present in several body fluids (Butler et al., 1980), which shows that its action is not restricted to the mammary gland. This paper describes the isolation and characterization of an integrin receptor for PAS-6/7 to which it binds in an RGD-dependent manner. Furthermore, the tissue distribution of PAS-6/7 and its ability to bind anionic phospholipids and MFGM lipids were investigated.

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[1] Abbreviations: PAS, periodic acid-Schiff reagent; MFGM, milk fat globule membrane; EGF, epidermal growth factor; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride.

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## EXPERIMENTAL PROCEDURES

*Identification of Proteins.* Protein samples were analyzed by SDS−PAGE, electroblotting, sequence analysis, and Western blotting as described (Benfeldt et al., 1995). Rabbit anti-bovine PAS-7 antibodies were raised (DAKO, Glostrup, Denmark) using purified bovine PAS-7 antigen (Hvarregaard et al., 1996). The immunoglobulin fraction was recovered from the immuno serum on a protein A Sepharose column and tested for purity. Identification of the $\alpha_V$ integrin subunit was performed using a mouse anti-human integrin $\alpha_V$ monoclonal antibody (Life Technologies, Roskilde, Denmark). Detection was performed with alkaline phosphatase conjugated immunoglobulins against primary antibody. Samples used for Western blotting of several tissues were prepared as follows: 100 $\mu$g of tissue was crushed in liquid nitrogen, homogenized in 3 mL of sample buffer with glass beads, and heated for 5 min at 95 °C followed by further homogenization. Afterwards, the sample was centrifuged and the supernatant was used for Western blotting. Bovine erythrocytes were washed twice in cold $H_2O$ to prepare ghost cells, and homogenized in 3 mL of sample buffer. Bovine plasma was diluted four times in sample buffer. A sample volume of 30 $\mu$L was loaded in each lane.

*Tissue Extracts and Protein Purification.* Tissue extractions were carried out essentially as described (Belkin et al., 1990). The entire procedure was carried out at +4 °C with exceptions specifically noted. Lactating bovine udder (2000 g) was homogenized in 4 L of deionized water containing 0.5 mM PMSF. After each step, centrifugation (10 min at 16000*g*) was performed to sediment material. The pellet was solubilized in 4 L of 20 mM Tris-HCl, pH 9.0, containing 1 mM EDTA and 0.5 mM PMSF, and the suspension was stirred for 1 h at 37 °C, followed by resuspension in 4 L of 20 mM Tris-HCl, pH 7.4, containing 0.6 M KCl and 0.5 mM PMSF, and stirred for 1 h. The pellet was washed in deionized water containing 0.5 mM PMSF, and centrifugation was repeated. The homogenate was resuspended in 2 L of extraction buffer, 20 mM Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM PMSF, and 0.5% reduced Triton X-100 and left with stirring overnight. The detergent solution was cleared by centrifugation (10 min at 16000*g*), and the supernatant was applied to an Amicon Concentration Cell with a Diaflo XM50 filter, and the volume was reduced to 200 mL. Purification of bovine $\alpha_V\beta_5$ integrin from the bovine udder extracts was accomplished by affinity chromatography on a 1.5 × 10 cm agarose column (Mini-Leak Medium, Kem-En-Tec, Copenhagen, Denmark) coupled with 50 mg of bovine PAS-6/7 prepared from bovine milk MFGM (Hvarregaard et al., 1996). The concentrated bovine udder extract was applied to the column, which was equilibrated in 20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1% reduced Triton X-100 (buffer A), and afterwards washed with 12 vol of buffer A. Elution of bound protein was performed either with 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, and 10 mM EDTA or with a synthetic tripeptide RGD (1 mg/mL) diluted in buffer A. Fractions were collected and concentrated on a Centricon-10 cell. Approximately 0.3 mg of integrin was purified from 2 kg of udder.

*Lipid Extract from MFGM.* Lipid was extracted from the MFGM with chloroform/methanol (2:1, v/v) as described (Kanno et al., 1975). To determine the amount of extracted MFGM lipid, 1 mL of extract was transferred to a microcentrifuge tube previously lyophilized for 24 h and tarred. The samples were lyophilized for 24 h, and the tubes were then weighed. To make certain that no protein was present in the extract and to quantify the amount of phosphatidylserine and phosphatidylethanolamine, amino acid analysis was performed.

*Solid Phase ELISA.* The solid phase ELISA for PAS-6/7 binding to phospholipid was performed as described (Bloom, 1987). L-2-Phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylinositol (PI), L-α-phosphatidic acid (PA), L-α-phosphatidyl-DL-glycerol (PG), sphingomyelin (S), cardiolipin (C) (Sigma Chemical Co., St. Louis), and MFGM lipid (MFGM-L) were tested. The assay was performed in triplicates. Phospholipid (100 $\mu$L) solutions of 3 $\mu$g/mL in methanol were added to micro well plates (Nunc, Roskilde, Denmark) and air-dried at room temperature. All subsequent steps were performed at 37 °C, and plates were washed three times in 0.2 M $NaH_2PO_4$, pH 7.0, 0.15 M NaCl, and 0.05% Tween-20. The plates were blocked with 200 $\mu$L of 50 mM Tris-HCl, pH 7.2, 0.15 M NaCl, and 0.05% (w/v) gelatine (blocking buffer). PAS-6/7 was diluted to the appropriate concentration in 20 mM Tris-HCl, pH 7.0, 0.15 M NaCl, and 0.05% (w/v) gelatine, and 100 $\mu$L was added per well. The plates were then incubated with 100 $\mu$L of polyclonal rabbit antibody against PAS-6/7 diluted in blocking buffer (0.5 $\mu$g/mL). Finally, plates were incubated with 100 $\mu$L of peroxidase-labeled swine anti-rabbit antibodies diluted 1:2000 in blocking buffer. Bound peroxidase activity was measured by adding 100 $\mu$L of 1,2-phenyleneamine dihydrochloride (DAKO, Glostrup, Denmark) dissolved in 12 mL of 0.1 M sodium citrate, pH 5.0. Reactions were stopped with 100 $\mu$L of 2 M $H_2SO_4$ after 5 min of incubation at room temperature. The absorbance at 490 nm was measured with a Bio-Tek EL 311s Autoreader (Bio-Tek, Winooski). Protein concentration in the PAS-6/7 stock was determined by amino acid analysis.

Solid phase ELISA was used as an integrin binding assay, and it was performed as described above, with the following modifications (Smith et al., 1990): 100 $\mu$L of integrin solutions (1 $\mu$g/mL) in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ were used for coating overnight at 4 °C and 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.5% gelatine (w/v) for blocking and dilution of PAS-6/7 and antibodies. As washing buffer, 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.05% Tween-20 (v/v) were used. RGD tripeptide (1 mg/mL) or EDTA (50 mM) was added in the PAS-6/7 incubation step. The assay was performed in triplicates.

## RESULTS

*Isolation of Bovine $\alpha_V\beta_5$ Integrin.* A sample of cell membranes was prepared from bovine udder tissue, and bound proteins were extracted with Triton X-100. The extracted proteins were applied onto an affinity chromatography column coupled with PAS-6/7, and bound protein was eluted with EDTA and analyzed by SDS−PAGE. Two distinct bands of approximately 93 and 139 kDa appeared

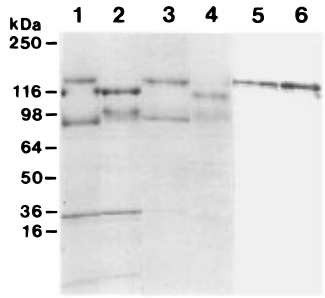


FIGURE 1: SDS−PAGE of $\alpha_V\beta_5$ integrin samples obtained by PAS-6/7 affinity chromatography. Lanes 1 and 2, EDTA-eluted protein; lanes 3 and 4, RGD-eluted protein; lanes 5 and 6 show a Western blot of EDTA- and RGD-eluted protein, respectively, incubated with anti-human $\alpha_V$ monoclonal antibody. Samples in lanes 2 and 4 were reduced. Molecular mass standards are indicated on the left.

**$\alpha_V$ heavy-chain**

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BOVINE:   FNLDVESPAEYSGPEGSYFG
HUMAN:    FNLDVDSPAEYSGPEGSYFG
MOUSE:    FNLDVESPAEYAGPEGSYFG
CHICKEN:  FNLDAERPAVYSGAEGSYFG
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**$\alpha_V$ light-chain**

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BOVINE:   DLTV(VE/XX)GDVXTL(G/X)XGIAEXL
HUMAN:    DLALSEGDIHTLGCGVAQCL
MOUSE:    GLTLREGDVHTLGCGIAKCL
CHICKEN:  DLTAIEGDVQTLGCGNADCL
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**$\beta_5$ chain**

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BOVINE:   LNIXTSGSATSXE
HUMAN:    LNICTSGSATSCE
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FIGURE 2: Alignment of N-terminal sequences of bovine $\alpha_V\beta_5$ integrin with other species. X indicates undetermined residues, and the Y/X notation is used when high yields indicate the presence of amino acid Y in both chain sequences, when sequencing of the unreduced $\alpha_V$ subunit. The presence of a less significant amino acid residue like histidine or cysteine, however, cannot be ruled out. ($\alpha_V$ subunit, accession no. P80746; $\beta_5$ subunit, accession no. P80747).

(Figure 1). When the same sample was analyzed under reducing conditions, the two bands moved closer to each other and appeared at approximately 106 and 127 kDa. To identify these two proteins, unreduced samples were subjected to electrophoresis and blotted onto a PVDF membrane, and N-terminal amino acid sequence analysis was performed on the excised bands. The protein appearing at 93 kDa gave a single sequence (Figure 2), whereas the 139 kDa band resulted in a double sequence, indicating that this band represented either two distinct proteins or two polypeptides linked with an intermolecular disulfide bond(s). Sequencing of the reduced 106 kDa protein gave the same sequence as the 93 kDa unreduced polypeptide, while the reduced 127 kDa protein was found to give a single amino acid sequence (Figure 2). This confirmed the above assumption suggesting the presence of a two-chained protein linked by a disulfide bond. The derived amino acid sequences were subjected to computer analysis, and they showed high similarity to human $\beta_5$ integrin subunit and the $\alpha_V$ integrin subunits from human, mouse, and chicken (Figure 2). Additionally, the identity of the 139 kDa band was confirmed in a Western blot using a monoclonal antibody against the human $\alpha_V$ integrin subunit (Figure 1).

The molecular mass of the $\alpha_V$ and $\beta_5$ subunits, as judged by SDS−PAGE, was consistent with previously reported data (Suzuki et al., 1986; Smith et al., 1990). The $\beta_5$ subunit appears to be heavily intra-disulfide-bonded and the $\alpha_V$ subunit has a heavy and light chain linked together by a disulfide bond, explaining the change in mass under reduction. Attempts to visualize the expected 25 kDa $\alpha_V$ light chain by overloading a SDS−PAGE followed by silver staining turned out negative; however, difficulties in visualizing this chain have been reported elsewhere (Suzuki et al., 1986).

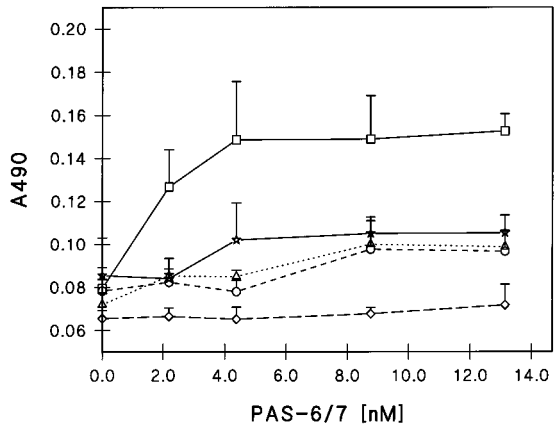


FIGURE 3: Solid phase ligand-binding analysis of purified $\alpha_V\beta_5$ integrin. Immobilized $\alpha_V\beta_5$ was incubated with various amounts of PAS-6/7 (□) or in combination with either RGD tripeptide (○), EDTA (△), RGD + EDTA (☆). Uncoated wells treated with PAS-6/7 were used as control (◇). Binding of PAS-6/7 was monitored with a polyclonal antibody raised against PAS-7.

Elution of integrin with an RGD tripeptide was performed to investigate whether the interaction between PAS-6/7 and the integrin was mediated by this cell adhesion motif displayed by PAS-6/7. Significant amounts of integrin were eluted with this tripeptide (Figure 1). The following EDTA treatment, however, also resulted in liberation of integrin as detected by SDS−PAGE (data not shown), indicating that the RGD interaction may not be solely responsible for the affinity to the $\alpha_V\beta_5$ integrin.

Another band was detected in the prepared integrin sample. This 35 kDa band was especially found in the EDTA eluted integrin fractions and only in trace amounts in the RGD-eluted integrin fractions (Figure 1). Several attempts to derive an N-terminal amino acid sequence failed, and the identification of this protein must await further analyses.

*Solid Phase Ligand-Binding Assay.* To further investigate the role of the RGD sequence motif of PAS-6/7 in the binding to $\alpha_V\beta_5$ integrin, micro well plates were coated with RGD-eluted integrin. Before coating, the integrin sample was subjected to dialysis to remove the RGD peptide, hereby excluding elution peptide interference in the assay. Subsequently, the wells were incubated with PAS-6/7 and effects on binding were investigated by including either RGD tripeptide, EDTA, or RGD together with EDTA. These experiments showed that binding of PAS-6/7 to $\alpha_V\beta_5$ was reduced by addition of the tripeptide (Figure 3). Furthermore, inclusion of EDTA reduced binding of PAS-6/7 to the same level, showing that ligand binding was dependent on the heterodimer conformation of the integrin. This result is consistent with the accepted model suggesting that the integrin complex is dependent on divalent cations and that the ligand-binding site might be situated in the cleft between the two subunits (Hynes, 1992). The structure of the integrin heterodimer will be disrupted upon addition of EDTA, whereby it looses its affinity toward RGD-interacting ligands (Gailit & Ruoslahti, 1988). Intriguingly though, treatment

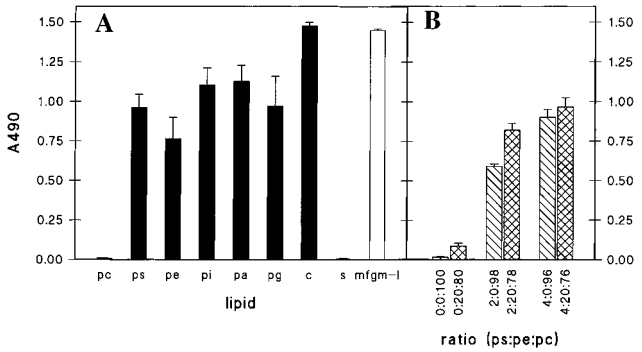


FIGURE 4: PAS-6/7 affinity for membrane derived lipids. Wells coated with lipid were incubated with PAS-6/7, and bound protein was monitored with a polyclonal antibody raised against PAS-7. Panel A: L-2-phosphatidylcholine (PC), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylinositol (PI), L-α-phosphatidic acid (PA), L-α-phosphatidyl-DL-glycerol (PG), cardiolipin (C), sphingomyelin (S), and MFGM lipid (MFGM-L). Panel B: Mixtures of L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylethanolamine (PE), L-2-phosphatidylcholine (PC). Molar ratios of the phospholipids are indicated under bars.

with neither RGD nor EDTA seemed to reduce the PAS-6/7 binding to background level. Therefore, the integrin sample was tested for trace amounts of PAS-6/7 originating from the tissue or from column leakage. This was determined by coating with the same amount of integrin and adding buffer instead of PAS-6/7 afterward. No reaction was detected, thereby ruling out that trace amounts of PAS-6/7 in the integrin sample could be responsible for the elevated background phenomena (data not shown). Similarly, no effect of Triton X-100 could be shown.

*PAS-6/7 Phospholipid Affinity.* The high degree of similarity between the C-terminal amino acid sequence of PAS-6/7 and the membrane associated C1−C2 domain of clotting factors V and VIII prompted an examination of the ability of PAS-6/7 to bind phospholipids in the same manner as these factors. To test this, micro well plates were coated with different purified membrane derived lipids and subsequently incubated with PAS-6/7. Since the amount of bound PAS-6/7 to the various lipids increased differently as a function of PAS-6/7 concentration, the concentration of PAS-6/7 (0.9 ng/$\mu$L) in the wells was held above saturation to enable comparison of PAS-6/7 binding. It was found that PAS-6/7 binds to phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, and cardiolipin, but not to phosphatidylcholine and sphingomyelin (Figure 4A). These data correspond well with similar studies on human factor VIII (Bloom, 1987; Gilbert & Drinkwater, 1993). Like factor VIII, PAS-6/7 has a preference for negatively charged lipids. To mimic PAS-6/7 association with the MFGM membrane, lipid extracted from the MFGM was included in the assay. The binding of PAS-6/7 was approximately 30% higher than observed with pure phospholipids, indicating that lipids of the MFGM display high affinity for PAS-6/7.

The binding of factor VIII to phospholipid membranes has been attributed to PS, an interaction which is enhanced by PE (Gilbert & Arena, 1995). The effect of PE in the binding of PAS-6/7 to phospholipids was investigated. Mixtures of PS, PE, and the nonbinding PC were made according to the reported levels of these in the MFGM (Jensen et al., 1991). Results showed that PE stimulated the binding of PAS-6/7, but far less than seen for factor VIII (Figure 4B). However, MFGM binding cannot exclusively be attributed to PS and PE since the used PS/PE/PC mixtures did not display the same level of binding as seen for MFGM lipids. Together, these data indicate that the MFGM contains a unique combination of lipids displaying high affinity for PAS-6/7.

*Tissue Distribution.* Extracts of 15 bovine tissues, erythrocyte ghosts, and plasma were prepared for Western blotting. Bovine PAS-6/7 was present in most tissues, and both resting and lactating mammary gland tissue showed high expression of PAS-6/7 (Figure 5). However, PAS-6/7 was not detected in the plasma and on erythrocyte membranes.

## DISCUSSION

As a member of the group of RGD binding integrins, the human $\alpha_V\beta_5$ integrin has been isolated and characterized (Suzuki et al., 1986; Smith et al., 1990). The function of the receptor is not known in detail, but several ligands bind to $\alpha_V\beta_5$, including vitronectin, the HIV Tat protein, and osteopontin, the first in an RGD-dependent manner (Smith et al., 1990; Vogel et al., 1993; Hu et al., 1995). Reports also indicate its participation in receptor-mediated endocytosis of vitronectin (Panetti et al., 1995). Integrin receptor $\alpha_V\beta_5$ is expressed in the human mammary gland, as detected by staining of human breast cancer cell lines and tissue-using antibodies against the human $\beta_5$ subunit, where it has been reported to be situated in the myoepithelial (Gui et al., 1995; Doerr & Jones, 1996). In contrast, PAS-6/7 has been found in the luminal epithelial and at the MFGM (Aoki et al., 1994).

Through ligand-binding assays it was examined whether the RGD sequence in PAS-6/7 was the sole origin of the binding. The results presented in this paper showed dependence on the RGD sequence, but the results were not conclusive. Functional $\alpha_V\beta_5$ integrin was obtained by elution with the RGD tripeptide. Nevertheless, a substantial amount of $\alpha_V\beta_5$ integrin was eluted afterward by EDTA indicating that small RGD peptides may bind the $\alpha_V\beta_5$ integrin with low affinity as seen for the $\alpha_V\beta_3$ integrin (Smith & Cheresh, 1988). This would explain the fact that PAS-6/7 binding to bovine $\alpha_V\beta_5$ integrin can be reduced with RGD, but not completely inhibited. Other modes of binding to the $\alpha_V\beta_5$ integrin have also been reported, e.g., the basic domain of HIV Tat protein and the human adenovirus coat protein Ad2 have demonstrated RGD- and cation-independent $\alpha_V\beta_5$ integrin binding (Vogel et al., 1993; Wickham et al., 1994), showing that ligand binding is possible for EDTA-treated integrin. Furthermore, it has been proposed that vitronectin uses the basic heparin-binding domain in synergy with the RGD sequence, since inhibition of vitronectin binding by the RGD peptide was difficult, requiring an 8-fold excess of peptide compared to other RGD-binding integrins (Cheresh et al., 1989). Another membrane glycoprotein, entactin, has been shown to bind different integrins involving two separate EGF domains (Dong et al., 1995). Entactin binds to $\alpha_V\beta_3$ through an RGD sequence situated in the third loop in one of the EGF domains and to a $\beta_1$ integrin family member with the other. The latter interaction is RGD independent. The tandem repeated EGF domain in PAS-6/7 could display binding to $\alpha_V\beta_5$ in a similar way with another binding site situated in the first EGF domain. However, this model is unlikely, since the recent cloning of BA46 has shown that only the RGD-containing EGF domain is present in the human protein (Couto et al., 1996). Although assay condi-

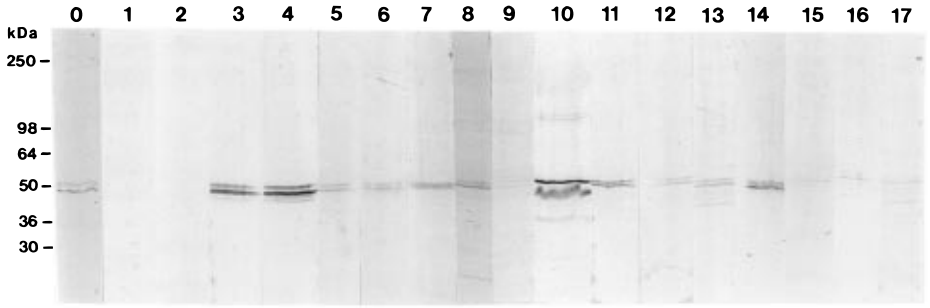


FIGURE 5: Western blots of tissue extracts. Lane 0, purified PAS-6/7; lane 1, plasma; lane 2, erythrocyte ghosts; lane 3, lactating mammary gland; lane 4, resting mammary gland; lane 5, thyroid gland; lane 6, lymph node; lane 7, salivary gland; lane 8, ovary; lane 9, pancreas; lane 10, heart; lane 11, lung; lane 12, abomasum; lane 13, spleen; lane 14, kidney; lane 15, liver; lane 16, colon; lane 17, ileum. Molecular mass standards are indicated on the left.

tions are different, it was found that cell attachment to BA46 could be completely blocked by addition of an RGD peptide (Peterson et al., 1995).

It has long been known that PAS-6/7 binds to membranes (Mather & Keenan, 1975), and recently, the orientation of membrane associated BA46 has been studied. By using epitope-mapped antibodies, it was shown that BA46 is attached on the cell surface with the C2 domain and the RGD-containing EGF domain exposed to the surroundings (Peterson et al., 1995). These data are consistent with the present study. A model of the C-terminal fragment of PAS-6/7 corresponding to the factor VIII α-helix led to the conclusion that binding of PAS-6/7 to MFGM and apical cell membrane could be facilitated by an amphipathic α-helix as well. However, two amino acids found in PAS-6/7 and the counterparts, His-397 and Arg-399, were not in agreement with the model of factor VIII. The His penetrates the membrane, introducing a positive charge to the hydrophobic face of the helix. This instability could be stabilized by the Arg, since it may interact with the phosphate moieties of phospholipids. The differences between the PAS-6/7 family and factor VIII could result in altered specificity for phospholipids. In the present study, PAS-6/7 was found to bind membranes with very low ratios of PS as compared to factor VIII, since much higher ratios of PS were needed for factor VIII binding (Gilbert & Arena, 1995). Addition of PE increased PAS-6/7 binding up to 35% (Figure 4B), and at higher ratios of PS, no effect of PE inclusion was observed (data not shown). This is in contrast to factor VIII, where the inclusion of PE has been shown to increase factor VIII binding by a 2−10-fold factor (Gilbert & Arena, 1995), which indicates that the effect of PE in this study is additive. Furthermore, a dendrogram made of all known C2-like domains placed BA46 (and PAS-6/7) with the C2 domain of factors V as the closest relative (Couto et al., 1996), and therefore lipid binding assays with factor V will be elucidative for the PAS-6/7 lipid specificity.

Since the $\alpha_V\beta_5$ integrin is found in a wide variety of tissues (Cheresh et al., 1989; Pasqualini et al., 1993; Gui et al., 1995), it was interesting to investigate the tissue distribution of glycoprotein PAS-6/7 as well. Originally, PAS-6/7 was described as an MFGM protein; however, nonlactating udder samples showed the same level of PAS-6/7 as the lactating udder. Investigation of a number of bovine tissues showed that the protein is widely distributed. Interestingly, PAS-6/7 was not detected in plasma and preparations of erythrocyte ghosts. The $\alpha_V\beta_5$ integrin has been reported to have been present in the blood vessel wall (Pasqualini et al., 1993), and it is tempting to speculate that the plasma is depleted for PAS-6/7 by binding to this integrin. Nevertheless, the plasma level of human BA46 has been found useful as a breast cancer maker, since it is overexpressed by breast cancer cells, resulting in an elevated level of BA46 in the blood (Salinas et al., 1987; Larocca et al., 1991). The normal level of BA46 has been reported to be approximately 25 ng/mL serum, which is close to the detection limit on a Western blot of PAS-6/7.

Data presented in this paper demonstrates the two-faced binding ability of PAS-6/7. Thus, PAS-6/7 may act as a linker between two cells by binding to the bovine $\alpha_V\beta_5$ integrin in one cell through the second EGF domain and to the cell surface of another cell with the C2 domain. Any function of PAS-6/7 can only be speculative, but the fact that it is widely present indicates some general function. Binding of PAS-6/7 to the $\alpha_V\beta_5$ integrin could result in a wide range of reactions such as proliferation and differentiation.

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