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In Vitro Interaction of a Polypeptide Homologous to Human Ro/SS-A Antigen (Calreticulin) with a Highly Conserved Amino Acid Sequence in the Cytoplasmic Domain of Integrin α Subunits†

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Received January 16, 1991; Revised Manuscript Received July 17, 1991

ABSTRACT: We endeavored to identify proteins interacting with KLGFFKR, a highly conserved motif in the cytoplasmic domain adjacent to the transmembrane domain of the α subunit of integrins. We found that affinity chromatography of cell extracts with this peptide followed by elution with EDTA resulted in the isolation of a 60-kDa protein (p60). The N-terminal amino acid sequence of this 60-kDa polypeptide was found to be highly homologous to the Ro/SS-A antigen, a 60-kDa protein homologous to calreticulin and Aplysia "memory molecule". The binding of p60 was found to be specific for the KLGFFKR sequence since this polypeptide did not bind to a peptide with a scrambled amino acid sequence (KLRFGFK), and it was also specifically eluted from the KLGFFKR affinity matrix ith soluble KLGFFKR peptide but not with the scrambled peptide. Solid phase in vitro binding assays demonstrated specific interaction of p60 with integrin α_3 and α_5 subunits but not with the β_1 subunit. Furthermore, p60 could be copurified with $\alpha_3\beta_1$ following coincubation in vitro. These interactions could be inhibited by KLGFFKR peptide and also by EDTA, indicating sequence-specific and divalent cation dependent binding. Despite the fact that calreticulin is thought to be localized in the endoplasmic reticulum, a pool of Ro/SS A antigen homologous 60-kDa polypeptide was found to be present in the soluble cytoplasm, indicating the feasibility of an interaction of p60 with the integrin α subunits. Our data suggest that p60 (Ro/SS-A Ag) can specifically bind to integrin α subunits via the highly conserved KLGFFKR amino acid sequence.

Integrins, a superfamily of cell surface receptors, provide a transmembrane link between the cell and the extracellular matrix (ECM) (Hynes, 1987; Ruoslahti & Pierschbacher, 1987). This transmembrane connection plays a central role in cellular adhesion, morphology, and migration. Included in this versatile family of integrins are the fibronectin, vitronectin, collagen, and laminin receptors. Some of the integrins interact with their respective ligands at a common tripeptide (Arg-Gly-Asp; RGD) recognition site. A typical integrin is a heterodimer consisting of an α and a β subunit in noncovalent association with one another. In some cases, the α subunit is composed of two disulfide-linked polypeptides that arise from proteolytic cleavage of a single precursor molecule. Amino

† This work was supported by grants to S.D. from the National Cancer Institute of Canada and the British Columbia Health Care and Research Foundation.

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0006-2960/91/0430-9859$02.50/0

Table I: Highly Conserved Amino Acid Sequence of the Integrin α-Subunit Cytoplasmic Domains Immediately following the Transmembrane Domain

vertebrate	α_1	-Lys-Ile-Gly-Phe-Phe-Lys-Arg-
	α_2	-Lys-Leu-Gly-Phe-Phe-Lys-Arg-
	α_3	-Lys-Cys-Gly-Phe-Phe-Lys-Arg-
	α_4	-Lys-Ala-Gly-Phe-Phe-Lys-Arg-
	α_5	-Lys-Leu-Gly-Phe-Phe-Lys-Arg-
	α_6	-Lys-Cys-Gly-Phe-Phe-Lys-Arg-
	αv	-Arg-Met-Gly-Phe-Phe-Lys-Arg-
	IIb	-Lys-Val-Gly-Phe-Phe-Lys-Arg-
	MacI	-Lys-Leu-Gly-Phe-Phe-Lys-Arg-
	p150	-Lys-Val-Gly-Phe-Phe-Lys-Arg-
Drosophila	PS2	-Lys-Cys-Gly-Phe-Phe-Asn-Arg-

acid sequence deduction suggests that each subunit of integrin consists of an extracellular domain, a membrane-spanning segment, and a short cytoplasmic domain (Hynes, 1987).

The initial classification of the integrins was based according to the β subunit they contained (Hynes, 1987; Ruoslahti & Pierschbacher, 1987), since it was believed that there were three β subunits (β_1, β_2, and β_3), each associating with multiple α subunits. However, with the isolation of more β subunits, it has become apparent that a single α subunit can associate with multiple β subunits (Cheresh et al., 1989; Hemler et al., 1989; Dedhar & Gray, 1990). Thus, although initially it was believed that the ligand specificity was mediated by α subunits, some β subunits may also determine this specificity. Since integrins function as transmembrane receptors that bridge the ECM to the cell, they presumably interact with various intracellular proteins. In recent years, several studies have suggested possible interactions between integrins and cytoskeletal proteins found in the focal contacts, sites at which the cell is in closest proximity to the ECM and where actin filaments are linked to the plasma membrane (Burridge et al., 1988). These include the interaction of integrin with talin (Horwitz et al., 1986) and the β_1 subunit of integrin with α-actinin (Otey et al., 1990).

To date, no attempt has been made to establish an interaction between the α subunit of integrins and proteins of the cell. The cDNAs of a number of α subunits have recently been cloned and sequenced (Argraves et al., 1987; Takada & Hemler, 1989; Takada et al., 1989; Tsuji et al., 1990; Tamura et al., 1990; Ignatius et al., 1990). Apart from a high degree of interspecies conservation of the amino acid sequence of cytoplasmic domains, as observed for human and murine α_5 subunit (Holers et al., 1989), the predicted amino acid sequence reveals a conserved stretch of seven amino acids in all the α subunits sequenced to date, including the *Drosophila melanogaster* PS2 antigen (Bogaert et al., 1987). As shown in Table I, this sequence, lysine-x-glycine-phenylalanine-phenylalanine-lysine-arginine (KXGFFKR), which immediately follows the transmembrane sequence, is highly conserved in all α subunits. Such a strict constraint on sequence conservation suggests an essential role of this motif for the structure and function of the integrin α subunits.

We have thus endeavored to isolate proteins that interact with this seven amino acid conserved sequence. Here we describe the isolation, by peptide affinity chromatography, of a 60-kDa polypeptide which binds specifically to the KLGFFKR sequence of integrin α subunits. This polypeptide was isolated from human osteosarcoma as well as human neuroblastoma cells. The N-terminal amino acid sequence of p60 was found to be highly homologous to rabbit calreticulin (Fliegel et al., 1989a) and the human Ro/SS-A antigen (McCauliffe et al., 1990a). We provide evidence demonstrating that this 60-kDa polypeptide interacts with the α subunit of integrins in a sequence-specific and divalent cation dependent manner, and discuss the implications.

Experimental Procedures

Materials. Cell lines SK-N-SH and HOS were obtained from the American Type Culture Collection (Rockville, MD) and were routinely cultured in DME medium supplemented with 10% heat-inactivated FBS. Monoclonal anti-α_3 antibody PIB5 was purchased from Telios (San Diego, CA). Anti-rabbit calreticulin was a generous gift from Dr. M. Michalak (University of Alberta, Edmonton). Antisynthetic peptide amino acid 6–19, corresponding to the N-terminal domain of the Ro/SS-A antigen (Lieu et al., 1988), was a kind gift from Dr. Sontheimer (Southwestern Medical School, University of Texas Health Science Centre, Dallas, TX). Anti-α-actinin and talin were kindly provided by Dr. K. Burridge (University of North Carolina, Chapel Hill, NC). Antibodies to actin, tropomyosin, and vinculin were purchased from Sigma Chemicals. Monoclonal anti-tubulin was purchased from New England Nuclear Products (Boston, MA). Horseradish peroxidase linked secondary antibodies were obtained from Amersham, Canada.

Integrin Receptor Purification. The integrin receptor $\alpha_5\beta_1$ was purified from ^{125}I surface-labeled human osteosarcoma cells by affinity chromatography on a column of fibronectin coupled to Sepharose as described by Pytela et al. (1987). The integrin $\alpha_3\beta_1$ was purified on an anti-α_3 immunoaffinity column. The purities of the integrins were determined by SDS–PAGE. For individual binding assay of α_3 and β_1, each subunit was electroeluted as described below for p60.

Affinity Chromatography. A seven amino acid peptide, KLGFFKR, corresponding to a highly conserved motif of the cytoplasmic domain of the α subunit of integrins and an altered order of the same sequence, KLRFGFK, were synthesized and purified by high-pressure liquid chromatography by the protein sequencing laboratory at the University of Victoria, British Columbia. Affinity matrices containing these peptides were prepared by coupling the peptides to CNBr-activated Sepharose (Pharmacia, Uppasala, Sweden).

Human osteosarcoma (HOS) or neuroblastoma (SK-N-SH) cell lines were harvested with 1 mM EDTA/phosphate-buffered saline (PBS) and washed twice with PBS, and the cells were extracted in wash buffer [25 mM HEPES buffer, pH 7.4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.15% Triton X-100] for 10 min on ice. Following centrifugation at 16000*g* for 15 min, the supernatant was either applied directly to the KLGFFKR–Sepharose affinity matrix or first applied to the scrambled-sequence peptide–Sepharose matrix and then to the KLGFFKR affinity matrix and allowed to bind to the matrix overnight at 4 °C. The column was washed extensively with wash buffer, and 0.5-mL fractions were eluted either with 20 mM EDTA (replacing the divalent cations in the wash buffer) or with wash buffer containing KLGFFKR or KLRFGFK peptides (1 mg/mL). Aliquots of each fraction were then electrophoresed on SDS–PAGE (Laemmli, 1970) and the protein bands visualized by staining with Coomassie brilliant blue R-250.

N-Terminal Sequence Analysis. A 10% SDS–polyacrylamide separating gel was prerun for 3 h prior to electrophoresis of the actual sample, and the stacker gel was prepared with agarose instead of polyacrylamide. The sample was electrophoresed in the presence of 100 mM sodium thioglycolate and 0.05 mM glutathione. Proteins were transferred to poly(vinylidene difluoride) (PVDF) microporous membrane and stained with Coomassie blue. The 60-kDa protein was se-

quenced for us by the Protein Sequencing Laboratory at the University of Victoria, British Columbia, using an applied biosystems protein microsequencer.

Western Blot Analysis. Samples from affinity column fractions were electrophoresed on 10% SDS–PAGE. The proteins from the gels were transferred to nitrocellulose, and Western blot analysis was performed according to the procedure of Towbin et al. (1979). Briefly, the nitrocellulose was incubated for 2 h in blocking buffer [2% BSA (bovine serum albumin) in TBS (150 mM NaCl/50 mM Tris-HCl, pH 7.4)], incubated for 90 min with the primary antibody (in blocking buffer). Following washing 4 times in TBS containing 0.05% Tween-20, the nitrocellulose was incubated for 90 min with horseradish peroxidase conjugated secondary antibody in blocking buffer and then washed again as above. The blots were finally developed with diaminobenzidine.

Isolation and Iodination of the 60-kDa Protein. Fractions eluted from the affinity column with 20 mM EDTA were pooled together, concentrated, and run on 10% SDS–PAGE. The exact position of p60 on the gel was determined by running an aliquot of the sample on a separate lane and staining with Coomassie blue. The p60 band was then cut out from the unstained gel and electroeluted into 12.5 mM Tris, 0.096 mM glycine, and 0.05% SDS overnight at 20 V. The protein was concentrated by using a Centricon microconcentrator (Amicon, Danver, MA) and the p60 concentration determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA). p60 in 400 μL of PBS was mixed with 100 μL of 1 mg/mL chloramine T in a glass vial, and 1 mCi of ^{125}I was added. The reaction was allowed to proceed for 30 min with mild agitation and stopped by adding 100 μL of 3 mg/mL metabisulfite. Labeled p60 was separated from the unbound label by applying the mixture onto a G-25M Sephadex column (Pharmacia) preequilibrated with PBS. The column was eluted with PBS, and the protein was further cleared of unbound label by placing the eluate on a microconcentrator and washing twice with PBS with final resuspension in PBS.

Solid Phase Binding Assay. Nontissue culture flat-bottom microtiter well plates (Linbro, McLeans, VA) were coated with either p60 or BSA at various concentrations (see legend of Figure 6) in PBS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM PMSF for 2 h at 37 °C. The unbound protein was removed, and the wells were blocked with 2% BSA in the above buffer for 30 min. The wells were then rinsed once with the above buffer, and the integrin receptor was added to the wells in the same buffer containing 0.3% Tween-20 and incubated for a further 2 h at 37 °C. The wells were washed 3 times with buffer plus Tween-20, the bound labeled material was extracted in PBS, 20 mM EDTA, 0.3% Tween-20, and 1% SDS, and ^{125}I cpm was counted in the γ scintillation counter.

To access the effect of EDTA on the interactions of integrins with p60, the assay was essentially the same as above except that various concentrations of EDTA were added simultaneously with integrins, and 0.001 mM EDTA replaced the cations in the buffer for the washings.

Copurification of p60 with $\alpha_3\beta_1$ from an Anti-α_3 Affinity Matrix. ^{125}I-Labeled integrin $\alpha_3\beta_1$ was incubated with ^{125}I-labeled p60 in TBS, 0.01% NaN_3, 2 mM PMSF, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, overnight at 4 °C with gentle rotation. The reaction mix was then applied to an anti-α_3 affinity column and the interaction allowed to proceed at 4 °C for 2 h. The column was washed extensively with column buffer (TBS: 2 mM PMSF, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.3% Tween-20). Preelution was with 10 mM $NaHPO_4$, pH 8.0, followed by elution with 100 mM $NaHPO_4$, pH 11.5. The pH of the eluate was immediately neutralized. The sample was electrophoresed on 7.5% SDS–PAGE, and the bands were visualized by autoradiography.

Immunofluorescence Studies. IMR-90 or HeLa cells were split, and 1×10^5 cells were added to each well of a 24-well tissue culture plate containing a glass coverslip and grown overnight. Coverslips were washed extensively with PBS and fixed in 2% paraformaldehyde, pH 7.2, for 1 h, 4 °C, except for tubulin preparations which were incubated for 5 min in 0.2 mg/mL dithiobis(succinimidyl propionate) followed by 5 min in 100 mM PIPES, pH 6.9, 1 mM EGTA, and 4% poly(ethylene glycol) 6000 prior to paraformaldehyde fixation. After being washed, cells were permeabilized in 0.1% Triton X-100 for 5 min. Coverslips were washed and incubated in preimmune rabbit serum, diluted 1:10, or in primary antibodies (anti-tubulin, undiluted or antisynthetic peptitide 6-19 corresponding to the N-terminal sequence of Ro/SS-A Ag diluted 1/10) for 1 h and then in secondary antibodies (anti-mouse conjugated to rhodamine; Tago Immunologicals, Burlingame, CA; or anti-rabbit FITC from BRL, Gaithersburg, MD, diluted at 1/50 and 1/100, respectively). Following 1-h incubation, they were mounted in mounting medium (Sigma) and sealed with clear nail polish. Indirect immunofluorescence was examined by using a Zeiss Axioskop microscope under oil immersion and photographed with Kodak T-Max film with an ASA of 400 pushed to 1600.

Preparation and Analysis of the HOS Soluble Cytoplasmic Fraction. HOS cells were resuspended in PBS containing 2 mM PMSF. The cells were disrupted by sonication at 20 kHz for 30 s at 4 °C (Branson Sonifier cell distrupter 350, Branson Sonic Power, Danbury, CT). The soluble cytoplasmic fraction was prepared by centrifugation at 140000*g* for 60 min at 4 °C in a Beckman Airfuge. The supernatant was removed, the protein concentration was determined, and an aliquot was analyzed by SDS–PAGE. The gel was transferred onto nitrocellulose and probed with an anti-Ro/SS A antigen antibody as described above.

Results

Affinity Isolation of KLGFFKR Binding Proteins. The seven amino acid sequence KLGFFKR, which corresponds to the common motif of the cytoplasmic domain of the α subunits (Table I), was synthesized, purified, and coupled to CNBr-activated Sepharose. The matrix thus prepared was used to isolate proteins which interacted with this sequence. Triton X-100 extracts of either HOS (human osteosarcoma) or SK-N-SH (neuroblastoma) cell lines were mixed with matrices in the presence of divalent cations followed by elution with EDTA. Figure 1 exhibits the elution profile of proteins released from the affinity matrix with buffer containing 20 mM EDTA, followed by SDS–polyacrylamide gel electrophoresis. A prominent band of relative molecular mass approximately 55–60 kDa was eluted from the column under these conditions (Figure 1, lanes 2 and 3). The isolation of this p60 protein is highly reproducible under these conditions.

Amino-Terminal Sequence of the 60-kDa Protein. Eluted proteins separated by gel electrophoresis were electrophoretically transferred to a poly(vinylidene difluoride) (PVDF) microporous membrane. The p60 protein band was identified by Coomassie blue staining and excised, and the N-terminal amino acid sequence was determined. The amino acid sequence of the first 18 residues was found to be highly homologous to human Ro/SS-A antigen (McCauliffe et al., 1990a) as shown in Figure 2, with only one amino acid difference at position 18 which is an arginine in the protein isolated here but a serine in Ro/SS-A antigen. Antibodies

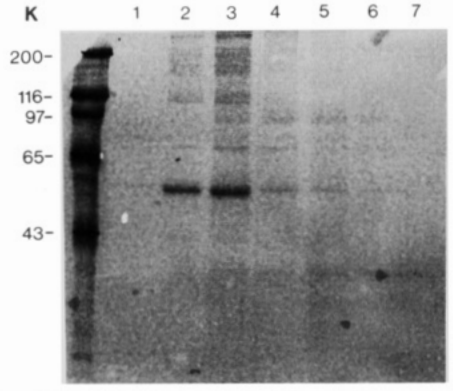

FIGURE 1: Affinity chromatography of the HOS Triton X-100 cell extract on KLGFFKR–Sepharose. Cells were extracted and affinity chromatogrphy was performed as described under Experimental Procedures. Proteins were separated on 7.5% SDS–PAGE (nonreduced). Lanes 2–7, aliquots of 0.5-mL fractions eluted with EDTA-containing buffer. Lane 1, molecular mass standards [myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), and BSA (67 kDa)].

NH_2-E P A V Y F K E Q F L D G D G W T R-------p60

NH_2-E P A V Y F K E Q F L D G D G W T S R-----Ro/SS-A Ag

FIGURE 2: Sequence comparison of the 18 N-terminal residues of p60 with the N-terminal sequence of the Ro/SS-A antigen.

to this protein are found in a number of autoimmune diseases, but the function of this protein remains undefined.

Specificity of the Interaction of p60 with the KLGFFKR Sequence. In order to determine whether the interaction of p60 with KLGFFKR was specific, an affinity matrix was prepared with a peptide with a similar amino acid composition but scrambled sequence, KLRFGFK. Figure 3 illustrates the differences in the elution of proteins by EDTA from affinity columns either containing control scrambled peptide, KLRFGFK (Figure 3A), or containing the KLGFFKR peptide (Figure 3B). As can be seen, a polypeptide with an approximate molecular mass of 50–60 kDa is eluted from the KLGFFKR column but not from the control KLRFGFK column. Utilizing an N-terminal peptide antiserum against human Ro/SS-A antigen, we were able to confirm by Western blot analysis of the eluted fractions from the two columns (Figure 3C) that this polypeptide is indeed the Ro/SS-A antigen homologous p60 identified in Figure 1. In addition, the Ro/SS-A antigen homologous p60 polypeptide is only present in the eluted fractions from the KLGFFKR column and is not detectable in the fractions from the control KLRFGFK column (Figure 3C).

We next determined if the p60 polypeptide could be displaced from the KLGFFKR affinity column by soluble KLGFFKR peptide. In this experiment, the cell extract was first absorbed onto the scrambled peptide affinity column sequence (KLRFGFK). The flow-through from this column was applied to the KLGFFKR column, which after extensive washing was eluted with soluble KLGFFKR peptide. Figure 4 (lane 3) demonstrates the presence of p60 in the KLGFFKR-eluted fraction as determined by Western blot analysis using an anti-calreticulin goat antiserum. p60 was not eluted by the control KLRFGFK peptide (Figure 4, lane 4). In addition to the presence of p60, the KLGFFKR eluates also consisted of talin and vinculin (Figure 4). Elution of the KLGFFKR column with soluble scrambled (KLRFGFK) peptide also resulted in the elution of talin and vinculin, but not p60, indicating that talin and vinculin do not bind specifically to KLGFFKR (data not shown). These experiments

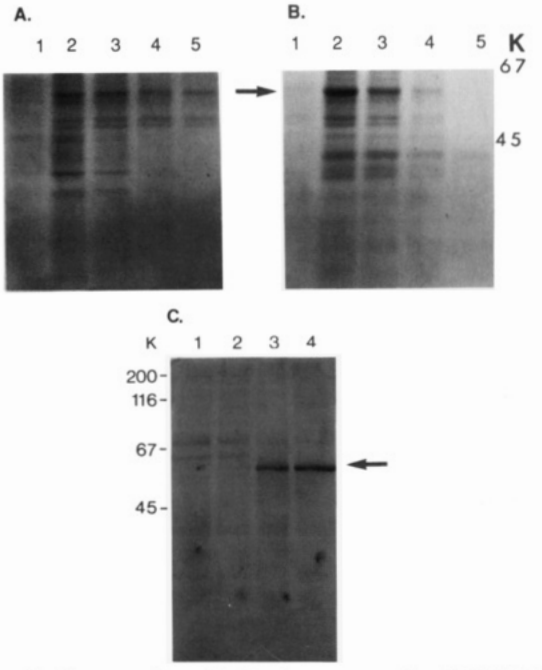

FIGURE 3: Comparative affinity chromatography of HOS Triton X-100 cell extracts on control KLRFGFK–Sepharose (A) and KLGFFKR–Sepharose (B). Fractions eluted with EDTA were analyzed by 7.5% SDS–PAGE under nonreducing conditions. Eluted fractions 2 and 3 from each column were electrophoretically transferred onto nitrocellulose and probed with a rabbit anti-human Ro/SS-A antigen antiserum (C) as described under Experimental Procedures.

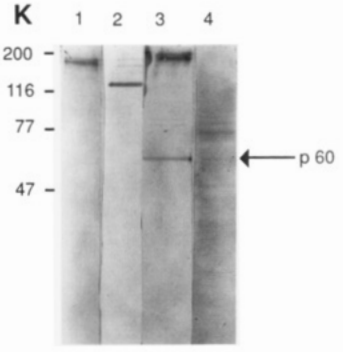

FIGURE 4: Western blot analysis of fractions eluted from the KLGFFKR–Sepharose column by soluble KLGFFKR or control KLRFGFK peptides. The proteins were anlyzed by 10% SDS–PAGE and electrophoretically transferred onto nitrocellulose. The blots were reacted with antibodies against talin (lane 1), vinculin (lane 2), and calreticulin (lanes 3 and 4) as described under Experimental Procedures. Fractions shown in lanes 1–3 were eluted with KLGFFKR peptide, whereas the fraction in lane 4 was eluted with the control KLRFGFK peptide. Anti-calrticulin cross-reactin p60 was eluted only with the KLGFFKR peptide (lane 3).

suggest that p60 binds specifically to the KLGFFKR sequence in a divalent cation dependent manner.

Interaction of p60 with Integrins in Vitro. To provide further evidence for the binding of p60 to the α subunits of integrins, an in vitro binding assay was used to determine the abilities of purified $\alpha\beta$-integrin heterodimers, as well as individual α and β subunits, to interact with p60 coated onto microtiter well plates. The p60 was purified by electroelution from gels and was greater than 95% pure as judged by SDS-PAGE and Coomassie blue staining. Figure 5 illustrates that $\alpha_5\beta_1$ (Figure 5A) and $\alpha_3\beta_1$ (Figure 5B) bind to p60 in a concentration-dependent manner. The integrins failed to bind to bovine serum albumin coated control plates. The data presented in Figure 5C demonstrate that the binding of integrins to p60 is α subunit specific, since purified β_1 subunit

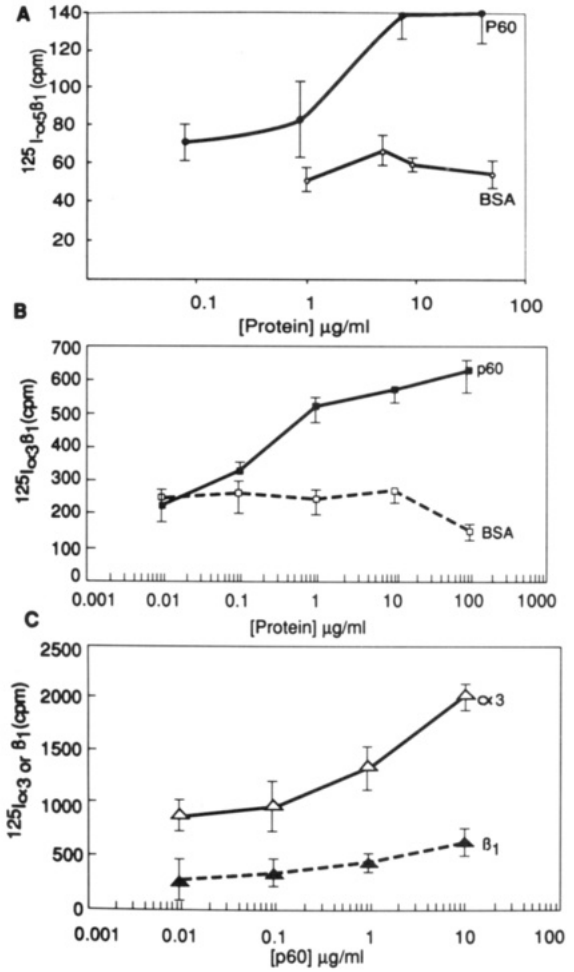

FIGURE 5: Solid phase binding assay with integrins. Microtiter wells were coated with various concentrations of either p60 or BSA, and the binding of ^{125}I-labeled integrins was determined as described under Experimental Procedures. (A) Interactions of p60 with ^{125}I-labeled $\alpha_5\beta_1$ heterodimer (the integrin was added at approximately 1600 cpm/well). (B) p60 interaction with ^{125}I-labeled $\alpha_3\beta_1$ heterodimer (added at 3000–4000 cpm/well). (C) p60 interaction with α_3 or β_1 (each added at 6000 cpm/well). Each figure is representative of three experiments, each done in duplicate.

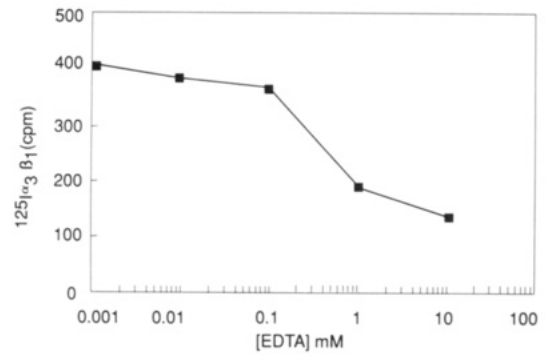

FIGURE 6: Inhibition of binding of integrin ($\alpha_3\beta_1$) to p60 with increasing concentrations of EDTA. The experimental protocol was similar to that described in Figure 4. The figure is representative of two experiments each done in duplicate.

was unable to bind to p60 whereas the α_3 subunit bound to p60 in a concentration-dependent manner. The binding of α_3 to p60 could be inhibited by EDTA (Figure 6), thus confirming that this interaction is divalent cation dependent.

The interaction of integrin α subunits with p60 was also demonstrated by copurification of p60 with $\alpha_3\beta_1$ from an anti-α_3 monoclonal antibody affinity column after coincubation

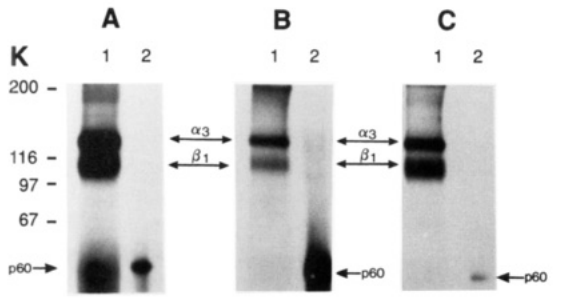

FIGURE 7: Copurification of p60 with $\alpha_3\beta_1$ from an anti-α_3 immunoaffinity matrix. ^{125}I-Labeled p60 was coincubated with ^{125}I-labeled $\alpha_3\beta_1$ in the presence or absence of either EDTA or KLGFFKR peptide, and the assay was carried out as described under Experimental Procedures. (A) Coelution of p60 with $\alpha_3\beta_1$ from an anti-α_3 column in the presenc eof divalent cations. Lane 1, eluate from the anti-α_3 matrix. Lane 2, ^{125}I-labeled p60 as marker indicatd by the arrowhead. (B) Inhibition of coelution of p60 and $\alpha_3\beta_1$ by 20 mM EDTA. Lanes 1 and 2, same as in (A). (C) Inhibition of coelution of p60 and $\alpha_3\beta_1$ by soluble KLGFFKR synthetic peptide

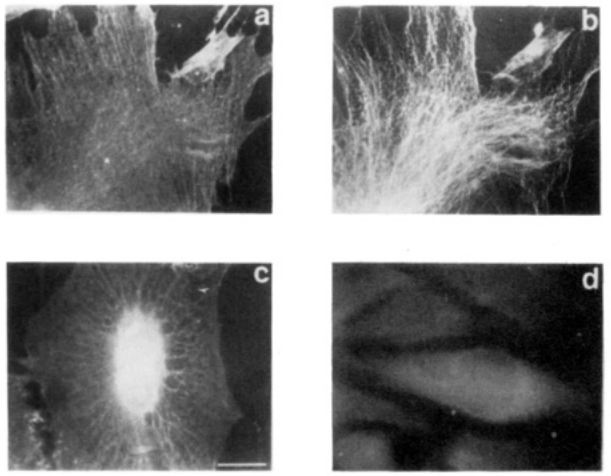

FIGURE 8: Immunofluorescent localization of p60 in IMR-90 (a and b) or HeLa epithelial (c) cells. Panel d shows staining of HeLa cells with preimmune rabbit serum used at the same dilution as the p60 antiserum. Costaining with antibodies directed against p60 (a) and tubulin (b) demonstrates that the p60 fibrillar structures stain differently than microtubules. Bar, 10 μm.

of $\alpha_3\beta_1$ and p60 in vitro (Figure 7A). This interaction was inhibited by EDTA (Figure 7B) as well as by soluble KLGFFKR peptide (Figure 7C), demonstrating that the interaction required divalent cations and occurred via the KLGFFKR sequence.

Cellular Localization of p60. Since p60 binds to integrins, we used immunofluorescence microscopy to determine the cellular localization of p60 in cultured cells. Previous studies on Ro/SS-A antigen and calreticulin indicated that p60 is an intracellular protein (Hendrick et al., 1981; Fliegel et al., 1989b). Figure 8 exhibits the distribution of p60 inside IMR-90 (Figure 8a) or Hela (Figure 8c) cells. The p60 staining exhibited both perinuclear and cytoplasmic localization, and the latter was in distinct fibrillar structures radiating from the nucleus to the cell surface. Such structures appear to be specific since these were not visible in preimmune control antiserum-stained cells (Figure 8d). Although these structures resembled microtubules, p60 is localized in a fibrillar network clearly distinct from that of microtubules (Figure 7B). Very little, if any, p60 was found to localize in focal adhesion plaques.

Because calreticulin has been hypothesized to be localized in the lumen of the endoplasmic reticulum (McCauliffe et al., 1990b), it is difficult to envisage an interaction between it and

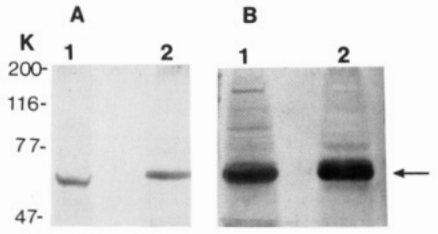

FIGURE 9: Western blot analysis for the presence of Ro/SS-A antigen homologous polypeptide in the cytoplasmic fraction of HOS cells. The cytoplasmic fraction was prepared as described under Experimental Procedures. An aliquot of this fraction (30 μg) was analyzed by SDS–PAGE, transferred onto nitrocellulose, and probed with anti-Ro/SS-A antigen antibody as described in Figure 3. (A) Experiment 1: lane 1, HOS soluble cytoplasmic fraction; lane 2, an aliquot of a fraction eluted from the KLGFFKR affinity column described in Figure 3C. (B) Experiment 2: lane 1, KLGFFKR affinity column isolated fraction; lane 2, HOS soluble cytoplasmic fraction. The arrow indicates the anti-Ro/SS-A antigen cross-reacting p60. Note that the slight difference in the apparent molecular weights of p60 in (A) is not present in (B). This difference may be due to the aberrant migration of p60 on SDS–polyacrylamide gels (McCauliffe et al., 1990a).

the cytoplasmic domain of the integrin α subunits in the intact cell. The notion that calreticulin is localized in the lumen of the endoplasmic reticulum is based mostly on the presence of the ccarboxyl-terminal KDEL sequence in the protein. This sequence has been shown to retain proteins in the endoplasmic reticulum. However, to our knowledge more direct physical evidence for the localization of calreticulin in the endoplasmic reticulum, e.g., by electron microscopy, is not available. Furthermore, the p60 polypeptide that we have isolated and shown to interact with the KLGFFKR cytoplasmic sequence of the integrin α subunits is homologous to calreticulin and Ro/SS-A antigen in the N-terminus of the protein. We do not know as yet whether this p60 is homologous to calreticulin over the entire amino acid sequence or that it contains the KDEL sequence. It is therefore possible that a pool of calreticulin homologous polypeptide exists in the soluble cellular cytoplasm. In order to determine whether this might be the case, we prepared a cytosolic preparation from HOS cells and carried out Western blot analysis of this preparation with the anti-Ro/SS-A antigen antibody. Figure 9 demonstrates that the soluble cytoplasmic fraction contains substantial levels of this antigen (Figure 9A, lane 1; Figure 9B, lane 2). Densitometric scanning of the Western blot together with the amounts of protein applied to the gels indicates that approximately 20% of the p60 is present in the cytosolic fraction. A more gentle method of lysing the cells, i.e., freeze–thawing, also resulted in the detection of cytosolic p60 (data not shown). Shown also in Figure 9 are aliquots of fractions eluted from the KLGFFKR column (see Figure 3), indicated here as a positive control. These results demonstrate that there indeed is a pool of cytoplasmic p60 which would therefore be able to bind to the KLGFFKR sequence of the integrin α subunits.

Discussion

This paper reports the isolation of proteins that interact with an amino acid sequence on the cytoplasmic domain of the α subunits of integrins. Our endeavor to search for proteins interacting with this domain was based on the fact that KLGFFKR is a highly conserved motif on all the α subunits sequenced to date including the PS2 antigen of *Drosophila* (Bogaert et al., 1987), suggesting that this sequence encodes an indispensable function. Our experiments have identified a polypeptide with an apparent molecular mass of 60 kDa which has an N-terminal amino acid sequence that is highly homologous (18 of the first 19 amino acid residues being identical) to the human Ro/SS-A antigen and rabbit calreticulin, and which specifically interacts with this motif in a divalent cation dependent manner.

The Ro/SS-A antigen is a cytoplasmic protein of unknown function. This antigen was originally described some 20 years ago in patients with systemic lupus erythematosus (Clark et al., 1969) and Sjogren's syndrome (Alspaugh & Tan, 1975) and has been detected in various human tissues including skin and heart (Deng et al., 1985). The Ro/SS-A antigen cDNA has recently been cloned and sequenced (McCauliffe et al., 1990a). It is a 48-kDa protein which, because of its high charge, migrates abberantly at approximately 60 kDa on SDS–polyacrylamide gels. The amino acid sequence of this protein includes a hydrophobic leader segment, a sequence for endoplasmic reticulum (ER) retention, and a calcium binding domain. This protein is the human equivalent of rabbit calreticulin, which is known to residue in the ER and the sarcoplasmic reticulum (SR) (Fliegel et al., 1989b). It has recently been shown that this protein is also found in mouse and *Drosophila* (Smith & Koch, 1989; McCauliffe et al., 1990b) and has a high degree of N-terminal homology to the Aplysia "memory molecule" p407, the expression of which is implicated in long-term memory acquisition (Kennedy et al., 1988). Thus, the presence of a high degree of structural conservation of this protein across species has prompted the authors to suggest that this protein has a basic cellular function which partially involves calcium binding.

Our data indicate that this may indeed be the case, that p60 is involved in a unified cellular function that involves interaction with integrins in a divalent cation dependent manner. We have been able to demonstrate a specific interaction bweteen p60 and integrin α subunits in four distinct ways. (i) p60 bound specifically, in the presence of divalent cations, to a KLGFFKR affinity matrix and could be displaced from this matrix by both EDTA and soluble KLGFFKR peptide. (ii) p60 did not bind to an affinity matrix prepared with the scrambled peptide KLRFGFK. The sequence specificity of this interaction was further confirmed by the inability of the KLRFGFK peptide to displace p60 from the KLGFFKR affinity matrix. (iii) The integrin α subunits α_5 and α_3, but not β_1, bound specifically and in a concentration- and cation-dependent manner, to p60. (iv) p60 could be copuified on an anti-α_3 affinity matrix after coincubation of purified $\alpha_3\beta_1$ with p60 in vitro. This interaction was inhibited by EDTA as well as by soluble KLGFFKR peptide, confirming the divalen cation dependency of the interaction and the sequence specificity. (v) A pool of Ro/SS-A antigen homologous 60-kDa polypeptide is present in the cytoplasm of HOS cells, making the possibility of a physiological interaction between p60 and integrins feasible.

Our failure to demonstrate colocalization of p60 with integrins at focal adhesions in immunofluorescence studies compels us to divorce the notion that p60 might be an anchoring protein involved in holding the α subunits of integrins to the cytoskeleton. Rather, we have shown p60 to be present in a perinuclear localization, as well as in fibrillar cytoskeleton-like structures which emanate from the nucleus and end up at the inner surface of the plasma membrane. Wester blot analysis of cytosolic preparations of HOS cells has demonstrated the presence of Ro/SS-A antigen homologous p60 polypeptide in the soluble cytoplasm of these cells.

The amino acid sequence of the recently cloned cDNAs of the Ro/SS-A antigen and the murine and rabbit calreticulin shows a hydrophobic leader segment indicating that this

protein is transported into the ER. These proteins also contain the carboxy-terminal sequence KDEL which may allow retention in the lumen of the ER. Being so, it is difficult to reconcile its interaction with the cytoplasmic domain of the α subunit of integrin. Therefore, it must be pointed out that antigenically different forms of the Ro/SS-A antigen have been reported (Rader et al., 1989) and presently it remains unclear whether these forms are functionally and structurally related. Furthermore, another recently cloned Ro cDNA sequence exhibits no similarity with the above-mentioned proteins (Deutscher et al., 1988). Thus, the Ro/SS-A form interacting with integrins could well be a form which is cytosolic, cytoskeletal, or retained in the membrane and not the lumen of the ER. This would explain the interaction of this protein with the cytoplasmic domain of the α subunit of integrins. Our finding that Ro/SS-A antigen homologous p60 is present in the cytoplasmic fraction of HOS cells gives added support for such an interaction. Furthermore, the Ro/SS-A antigen does contain some small hydrophobic regions, one of them proximal to the carboxy terminus which investigators suggest may be a membrane-spanning region (McCauliffe et al., 1990a). These proteins also have a highly acidic carboxy-terminal domain, and it has been speculated that this domain may represent a site through which the protein interacts with other proteins or the ER membrane via calcium bridges (Smith & Koch, 1989).

Having established the novel interaction between integrins and p60, several speculations can be made as to the role of this protein within the cellular environment. Since the conformational maturation of the integrin subunits and their assembly into heterodimers take place in the ER (Rosa & McEver, 1989), it is possible that the p60–integrin α-subunit interaction via the KLGFFKR sequence may be important during these processes. Recently, Volk et al. (1990) have demonstrated a role for integrins in the formation of the sarcomeric cytoarchitecture. This documentation is intriguing in light of the fact that calreticulin is also found in the SR of muscle cells and therefore may be anchoring integrins to the ER and SR membranes. Moreover, calreticulin is known to be a high-affinity calcium binding protein. One can thus speculate that this property may somehow be utilized in the signal transduction mechanisms of integrins.

Ro/SS-A antigen is also known to be an RNA binding protein, and ribonucleoproteins are known regulators of gene expression (Dreyfuss et al., 1988). To date, several ribonucleoproteins (RNP) have been assigned crucial functions; e.g., the signal recognition particle (SRP) is composed of six different polypeptides and a single RNA moiety and mediates the targeting of membrane and secretory proteins to the ER (Walter & Blobel, 1982). It is possible that Ro/SS-A antigen, which has four low molecular weight RNAs associated with it, has a crucial function within the cell involving regulation of integrin gene expression. Thus, several potential integrin-related roles can be envisaged for p60 within the cell, which could well involve regulation of integrin expression, anchorage in the ER and SR membranes, signal transduction assembly, and/or transport of integrins to the cell surface. Future direction will involve the elucidation of this function.

Acknowledgments

We thank Drs. Michalak and Sontheimer for their kind gifts of antibodies against rabbit calreticulin and human Ro/SS-A antigen, respectively, and Dr. Keith Burridge for his kind gift of antibodies against α-actinin and talin. We thank Linda Wood for secretarial assistance in the preparation of the manuscript.

Registry No. KLGFFKR, 136006-21-8; KLRFGFK, 136006-22-9.

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Interaction of Nitric Oxide with Ceruloplasmin Lacking an EPR-Detectable Type 2 Copper†

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Received October 19, 1990; Revised Manuscript Received July 8, 1991

ABSTRACT: Nitric oxide (NO) has previously been reported to modify the EPR spectrum of multicopper blue oxidases, disclosing a pure type 2 copper and inducing half-field transitions at $g = 4$. In the present work the reactivity of NO was reinvestigated with respect to ceruloplasmins having an apparently EPR-silent type 2 copper in their native state. The optical properties of NO-treated ceruloplasmin were independent of the initial redox state of the metal sites. Addition of NO caused the absorption at 600 nm to decrease in the case of oxidized ceruloplasmin and to increase when starting from the reduced proteins. In this latter case the absorbance at 330 nm was also restored, indicating that NO was able to reoxidize the reduced protein. In all cases the band at 600 nm leveled to ca. 60% of the intensity of the native untreated protein, and new bands below 500 nm appeared in the spectra. While the blue absorption band was restored by removal of NO, the absorbance below 500 nm remained higher even after dialysis. The EPR spectrum resulting from reaction of NO with either oxidized, partially reduced, or fully reduced ceruloplasmin consisted in all cases of a broad, structureless resonance around $g = 2$. NO caused the reversible disappearance of the type 1 copper EPR spectrum in oxidized ceruloplasmin. Also, the transient novel copper signal that arises during the anaerobic reduction process by ascorbate completely disappeared in the presence of NO and did not reappear upon removal of the gas. A type 2 copper signal and $g = 4$ transitions were observable upon addition of NO only in samples where type 2 copper was EPR-detectable before treatment with NO, as a consequence of prolonged storage of protein samples. Samples of this kind failed to reoxidize from their reduced state when reacted with NO. The results indicate that the integrity of the trinuclear cluster of ceruloplasmin, monitored by the absence of type 2 copper in the EPR spectrum of the native protein, modulates the behavior of the protein toward NO.

Ceruloplasmin is a copper-containing oxidase that is present in the plasma of vertebrates (Fee, 1975). It contains 5–7 prosthetic copper atoms that in vitro promote the reduction of oxygen to water coupled to one-electron oxidation of a variety of substrates. However, the physiological substrate is unknown and the role of this protein in vivo is still unclear. The possibility that ceruloplasmin acts as a copper-transport protein in the plasma cannot be discarded (Ryden, 1984).

Different forms of ceruloplasmin can be isolated depending on the purification procedure. Classical multistep protocols, widely used in the past, invariably produced a protein containing the three different types of copper, which characterize the multinuclear blue oxidases (ceruloplasmin, ascorbate oxidase, and laccase) (Fee, 1975): type 1, with a typical absorption band around 600 nm and an EPR[1] signal with a narrow hyperfine pattern; type 2, with an EPR spectrum typical of regularly coordinated tetragonal copper complexes; and type 3 copper, an EPR-silent antiferromagnetically coupled binuclear Cu(II)–Cu(II) unit absorbing at 330 nm. A general feature of those ceruloplasmins was that apparently one of the two blue ions was not involved in the four-electron transfer to oxygen, since it stayed reduced when oxygen was allowed to reoxidize the fully reduced protein (Ryden, 1984). More recently, procedures that permit a rapid isolation of the protein (Syed et al., 1982; Evans et al., 1985; Calabrese et al., 1988a, 1989; Musci et al., 1990) gave ceruloplasmins that showed, irrespective of their origin, different spectroscopic and

† This work was partially supported by CNR Special Project Chimica Fine 2 and by CNR P. F. Invecchiamento (publication code No. INV911029).
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[1] Abbreviations: EPR, electron paramagnetic resonance; pPD, *p*-phenylenediamine.

0006-2960/91/0430-9866$02.50/0