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BINDING OF THE LXCXE INSULIN MOTIF TO A HEXAPEPTIDE DERIVED FROM RETINOBLASTOMA PROTEIN

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Peptides corresponding to retinoblastoma protein (RB) fragment 649-654 (LFYKKV) were tested for their ability to recognize the **LXCXE** sequence motif in human papilloma virus type 16E7 protein (HPV-16E7) encompassing E7 residues 21-26 (**DLYCYE**) and an identical motif in human insulin comprising insulin B-chain residues 16-21 (**YLVCGE**), respectively. Interaction between these complementary peptide sequences was observed by several approaches, including direct and competitive ELISA as well as affinity chromatography. Moreover, we demonstrated that immobilized $RB_{649-654}$ displays specific recognition properties towards full-length insulin. Hence, this study provides a first experimental support for the previously anticipated complex formation between insulin and RB.

Retinoblastoma protein (RB) is a tumor suppressor gene product which appears to play a central role in the transcriptional control of cell differentiation and proliferation [1]. Consequently, it is a common target for a variety of proteins with growth regulatory activity. Among these RB-complexing molecules are viral oncoproteins [2,3], and cellular ligands [4], which share the sequence motif LXCXE shown to be critical for binding of these proteins to RB. A recent study has suggested that insulin and retinoblastoma protein may interact with one another [5]. This prediction was derived from the identification of an amino acid sequence of the form LXCXE in the B-chain of insulin [5]. Consistent with this structural relationship, insulin has already been implicated in a mitogenic pathway putatively involving intracellular associations with as yet unknown transcription factors [6]. Moreover, the proposed interaction between the B-chain of insulin and RB has added to the concept of intracrine cell growth regulation and raised the intriguing possibility of intracellular signalling through protein subunits, particularly under the conditions of embryogenesis and oncogenesis [7]. An RB binding site common for the various LXCXE motifs found in viral and host proteins, including insulin, has previously been deduced through the hydropathic complementarity approach (8). This procedure entails searching for binding sites with opposite hydropathic character in proteins shown or proposed to assemble with one another and relies on the

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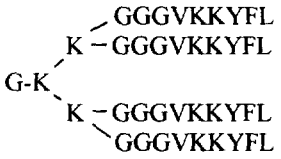
large body of evidence supporting the concept of inverse hydropathy as a major driving force in protein-protein interactions (9), protein folding (10) and anti-idiotypic interactions between antibodies (11). The foundation of this methodology stems from the observation that codons for hydrophilic amino acids on one strand of DNA are complemented by codons for hydrophobic amino acids on the other strand, and vice versa (12). In an extension of this characteristic of the genetic code, it has been shown that RNA stretches transcribed from opposing DNA segments or specified by complementary DNA domains encode peptides with inverse hydropathic profiles and that these peptides bind one another with considerable affinity (13). Using this approach, a putative RB binding site common for the various LXCXE motifs has been proposed to encompass residues 649-654 of RB or LFYKKV, respectively (5,8). Furthermore, a sterically constrained variant of an oligopeptide comprising the RB 649-654 sequence and $RB_{649-654}$ itself have been suggested as candidate agents for *in vivo* treatment against various tumors (5,7). This has been derived from the scenario according to which either of these peptides may associate with the LXCXE sequence of human papilloma virus type 16 E7 protein (HPV-16 E7) or insulin and thus prevent these growth-promoting molecules from employing their LXCXE sequence in the binding and inactivation of RB (5,7). In this study we have examined in vitro the proposed interaction between the $RB_{649-654}$ fragment and HPV_{21-26} or $insulin_{16-21}$ respectively, using direct and competitive ELISA, and affinity chromatography.

MATERIALS AND METHODS

Peptide synthesis, purification, and characterization. Peptides have been produced by solid phase peptide synthesis following the Fmoc methodology using a fully automated ABI 431 A peptide synthesizer. After cleavage from resin, peptides have been purified from contaminants by RP-HPLC on a semipreparative column. Peptide identity has been confirmed by amino acid analysis of acid hydrolyzates and by TOF-MALDI mass spectrometry. Peptide corresponding to the RB fragment 649-654 [LFYKKV] has been produced in a tetrameric form starting from a tetradentate lysine core introducing a C-terminal triglycine spacer, leading to the final structure:



4RB

Peptide biotinylation. Peptides or insulin (Sigma) were dissolved in sodium acetate 0.1 M pH 6.5 (2 mg/ml) and 1mg of biotin-ε-aminocaproate-N-hydroxysuccinimide ester dissolved in water/methanol 1:1 was added. Extent of biotin incorporation has been checked by RP-HPLC. At the end, derivatized product has been purified from residual reagents and contaminants by semipreparative RP-HPLC.

Solid phase assays. Microtiter plates (NUNC) were incubated with 0-50 μg/ml of tetrameric RB solutions (50 μl/well) in 0.1 M sodium carbonate buffer, pH 9.6, overnight at room temperature. After coating, the plates were first washed four times with PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.3) and then 200 μl of PBS containing 3% BSA (PBS-B) were added to each

well to block the uncoated plastic surface. The plates were then washed again four times with PBS containing 0.05% Tween 20 (PBS-T) and filled with samples containing biotinylated peptides at varying concentrations (0-10 μg/ml). After 1 h incubation, the plates were washed eight times with PBS-T, and wells were filled with 100 μl of a streptavidin-peroxidase solution diluted 1: 100 with PBS-T. After 1 h, plates were washed again and wells filled with chromogenic substrate. For competition experiments, microtiter plates coated with tetrameric RB were treated with solution containing biotinylated peptide (1-2 μg/ml) and unlabeled tetrameric RB (0-100 μg/ml). The absorbance at 490 nm of each well was determined, and the competitor concentration was plotted against the percentage of bound labelled peptide (B/Bo).

Affinity chromatography experiments. Tetrameric RB peptide (5 mg) has been dissolved in 5 ml of 0.1 M sodium bicarbonate pH 8.5 and added to 1.2 g of EUPERGIT C30 N (Rohm & Haas), epoxy activated methacrylic support for affinity chromatography. Extent of peptide immobilization has been followed by RP-HPLC monitoring of the incubation mixture at different times. Almost 90 % of the initial amount of added peptide was immobilized after 24 hours incubation. Unreacted epoxydes were deactivated by resin treatment with 1 M TRIS pH 9.0 for 2 hours. At the end peptide- derivatized resin has been packed in a 2.3 ml glass column (Omni), and equilibrated at a flow rate of 1.0 ml/min with 50 mM TRIS pH 7.0. A 200 μl sample containing insulin (0.1 mg/ml) and a mixture of unrelated proteins and peptides (2 mg/ml total) has been injected in to the column, and after elution of unbound material, the eluent has been changed to 0.1 M acetic acid. Fractions corresponding to the bound and unbound material have been collected for RP-HPLC analysis.

RESULTS

Peptide binding monitored by ELISA assays. The previously proposed interaction between RB and an HPV or insulin fragment [5,7,8] has been analyzed by solid phase assays on microtiter plates coated with increasing amounts of 4RB. As shown in Figure 1A, binding of biotinylated HPV peptide to immobilized tetrameric RB was observed in a way linearly dependent on the amount of tetrameric peptide used in the coating procedure and on the amount of biotinylated peptide . Binding to uncoated plates was negligible (O.D. < 0.1). Binding was specific, since presence of

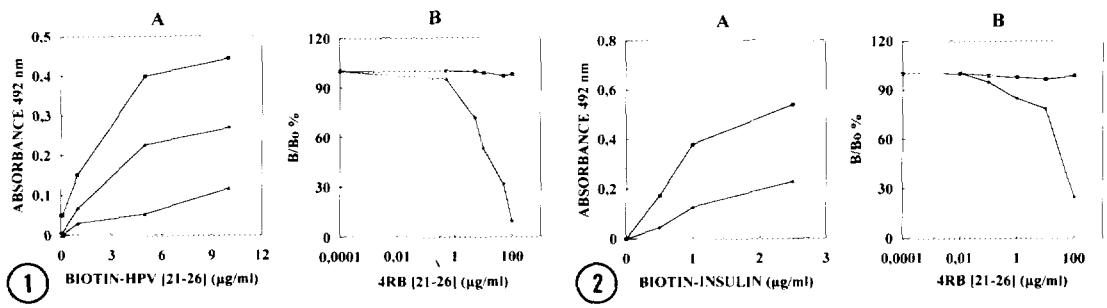


Figure 1. **A**: Binding of biotin-HPV [21-26] to microtiter plates coated with 4RB at concentration 1 μg/well (▲), 2.5 μg/well (◆) and 5 μg/well (■). **B**: Competitive binding of biotin-HPV [21-26] fragment to microtiter plates coated with 4RB in the presence of varying amounts of 4RB (◆) or unrelated peptides (■).

Figure 2. **A**: Binding of biotin-insulin to microtiter plates coated with 4RB at concentration 5 μg/well (■) and 1 μg/well (◆). **B**: Competitive binding of biotin-insulin to microtiter plates coated with 4RB in the presence of varying amounts of 4RB (◆) or unrelated peptides (■).

tetrameric RB peptide in the binding solution inhibited the interaction in a dose dependent manner, while BSA or unrelated peptides did not exhibit any effect (Figure 1B). Similar selectivity was shown for biotynilated insulin fragment 16-21. Also in this case binding was linearly dependent on the amount of tetrameric RB in the plate and on the amount of biotinylated peptide. Furthermore, binding was inhibited in the presence of soluble 4RB peptide (not shown). In order to further confirm the occurrence of recognition between RB fragment and insulin, experiments were carried on microtiter plates coated with tetrameric RB and biotinylated insulin. As shown in Figure 2 A and B, also in this case binding was observed with the same degree of selectivity and specificity as before.

Affinity chromatography on immobilized tetrameric RB fragment.

Further evidence of interaction specificity between 4RB and insulin was shown by affinity chromatography experiments with columns prepared by immobilizing 4RB on a preactivated support. A mixture containing several unrelated proteins and peptides (total concentration 2 mg/ml) was spiked with insulin (0.1 mg/ml final concentration) and applied to the column equilibrated with 50 mM TRIS pH 6.8. After elution of unbound material, the eluent was changed to 0.1 M acetic acid to elute bound material (Figure 3A). Fractions corresponding to the unbound and bound material were collected for RP-HPLC analysis. As shown in Figure 3B, while the unretarded material contained the majority of unrelated peptides and proteins but no insulin, the latter was recovered in the bound material essentially free from contaminants. This further supported the contention of binding specificity and selectivity between 4RB and insulin, and at the same time demonstrated the usefulness of immobilized 4RB for affinity purification of insulin.

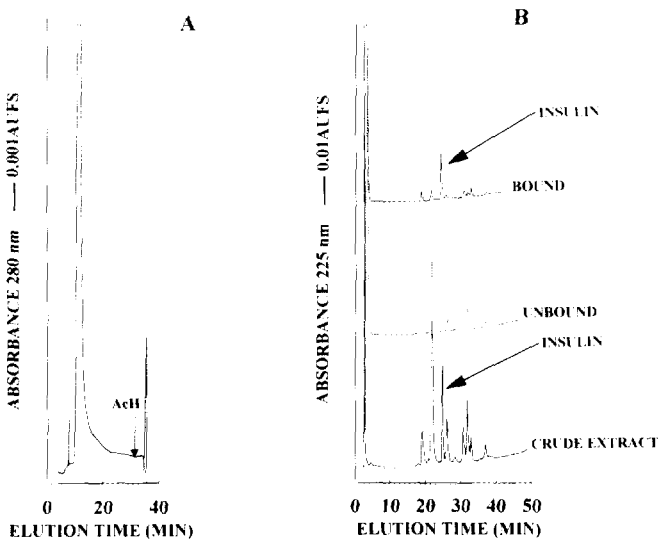


Figure 3. **A**: Affinity chromatography of protein/peptide mixture containing insulin on the 4RB column, equilibrated at a flow rate of 1 ml/min with 50 mM TRIS,pH 6.8. At the position indicated by the arrow, the eluent was changed to 0.1 M HAc. **B**: RP-HPLC analysis of crude peptide/protein mixture containing insulin (bottom), unbound material peak 1 (center) and bound material peak 2 (top).

DISCUSSION

Dissecting the structure-function relationships inherent in the sequence and conformation of the tumor suppressor retinoblastoma protein (RB) may represent an important avenue towards understanding the intimate involvement of this molecule in negative growth regulation and cell cycle progression as well as help devise small RB-derived molecules that display in vivo properties compatible with an efficient agent for antineoplastic treatment. Recent additions towards these goals are the identification of distinct sites on RB such as a nuclear localization sequence (14), a carboxyterminal growth suppressor domain (15), an amino terminal dimerization region (16) and the prediction of an RB binding site for the LXCXE sequence motif of growth-promoting viral and host proteins (5,7,8) based on the hydropathic complementarity approach.

The present study has provided experimental evidence for the previous prediction according to which $RB_{649-654}$ is a potential RB binding site for various proteins containing the LXCXE motif (5,7,8) showing that the LXCXE motif of HPV-16 E7 (HPV-16 $E7_{21-26}$) binds $RB_{649-654}$ with measurable affinity. It remains to be determined whether this viral peptide binds the same sequence when confronted with full-length RB and also whether $RB_{649-654}$ participates *in vivo* in the association of RB with E7. Moreover, we have found that $RB_{649-654}$ is able to specifically recognize the LXCXE motif of insulin (insulin B-chain 16-21), providing the underlying principle for the design and successful application of immobilized tetrameric $RB_{649-654}$ for the affinity purification of insulin. Accordingly, the preceding sequence analysis of RB and insulin has been confirmed as a useful tool in identifying binding sites for a possible interaction between these two molecules that were previously not known to interact with one another.

Hence, it appears that such interactions governed by opposite hydropathic profiles of the interacting sites as postulated by the hydropathic complementary approach (13) are specific enough for affinity purification, thus confirming earlier data in this field (9), and emphasising that such small peptides could be an interesting alternative to other approaches including those based on antibodies that generally are superior in affinity but have no apparent relative advantage in specificity. Furthermore, this type of peptide-based approaches are more convenient and less expensive than conventional methods. For instance, the $RB_{649-654}$ tetrameric peptide immobilized on solid supports may simplify current approaches for the isolation of recombinant insulin fusion protein from E. coli (17).

It remains to be seen in future in vitro and *in vivo* studies whether $RB_{649-654}$ is able to substitute for the full-length RB protein in its interaction with the LXCXE motif of various growth regulators and distinguishes between subtle sequence variants such as **LXCXE** in HPV-16 E7 (3) vs. **LXEXE** in the RB-binding transcription factor E2F (18). It would be intriguing if, besides its usefulness in the purification of insulin shown in the present work, $RB_{649-654}$ or a derivative thereof further validated its previously predicted clinical potential (7,8) by emerging, in the course of further explorations, as an efficient molecule for the treatment of neoplasia and thus added to the importance of a structure based approach in drug discovery.

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