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Isolation and Characterization of Monoclonal Antibodies Specific for the Cardiac Muscarinic Acetylcholine Receptor†

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ABSTRACT: A number of monoclonal antibodies were raised against the purified porcine atrial muscarinic acetylcholine receptor. The antibodies were shown to exhibit a high degree of specificity for the receptor by their ability to recognize the purified receptor but not other porcine atrial glycoproteins in enzyme-linked solid-phase immunosorptive assays and by immunoblot analyses. Several of the antibodies were able to quantitatively precipitate the muscarinic receptor in both pig and rat heart and a portion of the receptor from rat cerebellum but little if any receptor from rat cerebral cortex. Thus, these monoclonal antibodies not only exhibit specificity for the muscarinic receptor but also are specific for the cardiac receptor subtype.

Muscarinic acetylcholine receptors (mAChR)[1] are present in heart and smooth muscles, in neurons in the central and peripheral nervous system, and in a variety of exocrine glands. While most common muscarinic receptor ligands bind with equal affinities to mAChR in different tissues, the existence of pharmacologically distinct subclasses of mAChR can be demonstrated. Thus, both ligand binding techniques and physiological studies have demonstrated that the antagonist pirenzepine and the agonist McN-A-343 have low affinity for the mAChR in cardiac and smooth muscle, in exocrine glands, and in some regions of the central nervous system and have significanty higher affinity for the mAChR in sympathetic ganglia and certain brain regions (Hammer et al., 1980; Hammer & Giachetti, 1982; Wess et al., 1984). Muscarinic receptors with high affinity for pirenzepine have come to be called M_1 muscarinic receptors and those with low affinity, M_2 receptors. In addition, the antagonist AF-DX 116 can distinguish between different classes of receptors with low affinity for pirenzepine, having a higher affinity for the M_2 receptor in the heart than for the M_2 receptor in certain glands (Hammer et al., 1986; Giachetti et al., 1986; Michaletti et al., 1987). AF-DX 116 can also differentiate between different classes of M_2 receptors in the central nervous system (Giraldo et al., 1987).

Initial biochemical studies on the mAChR did not detect differences between the mAChR from different tissues. For example, affinity labeling of receptors followed by either SDS gel electrophoresis or partial proteolytic peptide mapping did not demonstrate the existence of major structural differences in different tissues (Birdsall et al., 1979; Venter, 1983). In addition, purification of the mAChR from porcine brain and heart yielded preparations of similar polypeptide composition (Peterson et al., 1984; Haga & Haga, 1985). However, determination of the primary structures of the M_1 receptor from porcine brain (Kubo et al., 1986a) and the M_2 receptor from porcine atria (Kubo et al., 1986b; Peralta et al., 1987) by cDNA cloning indicated considerable structural diversity with only 38% amino acid homology. In addition, the results of Northern blot hybridization in different brain regions (Kubo et al., 1986a,b) when combined with ligand binding studies (Giraldo et al., 1987) suggest that the glandular receptor may also be structurally distinct.

Studies of the nicotinic AChR have been greatly assisted by the availability of libraries of mAbs specific for the nAChR, which have been of great value in research on the regulation of synthesis and assembly of the receptor (Merlie, 1984; Smith et al., 1987), as well as in studies on its structure and transmembrane orientation (Lindstrom et al., 1984; LaRochelle et al., 1985; Ratnam et al., 1986). There have been relatively few immunological studies of the muscarinic AChR. Venter et al. (1984) reported the isolation of several monoclonal antibodies (mAb) against the mAChR, some of which also cross-reacted with α-adrenergic receptors. However, these antibodies were isolated following immunization with a crude immunogen and evidence of their specificity was not presented, so it is possible that these mAbs may recognize epitopes, such as carbohydrate, which are present on many membrane proteins. Andre et al. (1984) reported the isolation of two mAbs that appeared to be specific for the mAChR on the basis of immunoblot and immunoprecipitation analysis. Lieber et al. (1984) showed that these antibodies possessed muscarinic agonist-like activity in intact tissues that could be blocked by the muscarinic antagonist atropine, suggesting that these antibodies had a high degree of specificity for the muscarinic receptor. Peralta et al. (1987) have reported the isolation of

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[1] Abbreviations: mAChR, muscarinic acetylcholine receptor; PrBCM, propyl benzilylcholine mustard; SDS, sodium dodecyl sulfate; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorptive assay; QNB, *l*-quinucidinyl benzilate; Ig, immunoglobulin; nAChR, nicotinic AChR; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

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a polyclonal anti-synthetic peptide antiserum directed against a known muscarinic receptor specific sequence that could immunoprecipitate the cardiac mAChR.

To aid in our studies on the mAChR, we have used purified preparations of the porcine cardiac mAChR to isolate mAbs specific for the muscarinic receptor. We report here the isolation of a panel of monoclonal antibodies that specifically recognize the muscarinic receptor from porcine atria. Some of these mAbs cross-react with the mAChR from rat heart and distinguish the cardiac and brain receptors.

Materials and Methods

Purification of mAChR. The mAChR was purified from porcine atria as previously described (Peterson et al., 1984). Partially purified preparations (5–10% pure) of mAChR for use in ELISA assays were obtained by use of this procedure up to the affinity column step. Preparations of pig heart glycoproteins not containing the mAChR were obtained from side fractions of the DEAE column step.

Immunization and Fusion. All of the anti-receptor mAbs described here resulted from the immunization of a single mouse and subsequent fusion. Purified mAChR (4 μg, in 0.05 mL of 0.08% digitonin, 0.016% cholate, 25 mM imidazole, and 1 mM EDTA, pH 7.4) was emulsified with 0.15 mL of adjuvant (MPL + TDL emulsion, Ribi Immunochemical Research, Inc., Hamilton, MN) and injected intraperitoneally into a Balb/c mouse. The mouse was boosted with 2 μg of purified receptor in the same adjuvant at intervals of 2, 4, and 2 weeks. The mouse was bled 3 weeks after the first booster injection, and the presence of anti-receptor antibodies was confirmed by immunoblot analysis (data not shown). The spleen was removed 3 days after the final booster injection, and spleen cells were fused with SP-2 myeloma cells by the procedure of Galfrey et al. (1977) as modified by de St. Groth and Scheidegger (1980) to minimize the possibility of multiple hybridoma clones arising in a single well. Clones were tested by ELISA for production of anti-mAChR antibody starting at 14 days after fusion.

For production of ascities fluid, hybridoma cells were preadapted for growth in vivo by subcutaneous injection of (3–10) × 10^5 cells into the back of a Balb/c mouse. After 2–3 weeks, the cells were harvested and grown as ascites tumors in pristane-primed mice as described by Galfrey et al. (1977).

ELISA. ELISA using partially purified and purified mAChR as target antigens were carried out by a method analogous to that described by Casadei et al. (1984) for detergent-solubilized sodium channels. In brief, microtiter plates were precoated with polylysine (0.25 μg/well) and then incubated with either partially purified or purified mAChR (0.025–0.05 μg/well). Culture medium (50 μL) and goat anti-mouse Ig(G,A,M) conjugated to alkaline phosphatase were then added, and ELISA assays were performed as described by Gainer and Nathanson (1986). Initial screening of hybridoma wells was performed with partially purified (5–10% theoretical specific activity) mAChR preparations as the target antigen. Positive wells were rescreened with highly purified (>95% theoretical specific activity) preparations of the receptor as the target antigen (0.025–0.05 μg/well). Nonreceptor-containing glycoprotein preparations from pig atria were used in control experiments at the same protein concentrations as the purified receptor preparations.

Subtype Analysis. The subtype of the mAbs produced by the hybridoma cells was determined by Ouchterlony immunodiffusion using a typing kit from Miles as described in the instructions provided by the manufacturer.

Immunoprecipitation Analysis. For immunoprecipitation of the solubilized porcine atrial mAChR, atrial membranes were purified as described by Peterson and Schimerlik (1984) and stored at −80 °C. After thawing, membranes were washed twice in 50 mM NaH_2PO_4 (pH 7.4), resuspended at a protein concentration of 6–7 mg/mL in solubilization buffer (1% digitonin, 0.1% cholate, 10 mM KH_2PO_4, pH 7.0, 1 mM EDTA, 50 mM NaCl), mixed in the cold for 1 h, and centrifuged at 15000 rpm for 30 min in a Beckman JA-20 rotor. For solubilization of mAChR from rat heart and rat brain, the tissue was homogenized, crude membrane preparations were isolated essentially as described previously (Halvorsen & Nathanson, 1981; Liles et al., 1986), and the receptor was then solubilized as described above.

Rabbit anti-mouse immunoglobulin (heavy and light chain specific) Immunobeads (Bio-Rad) were washed twice in PBS and then twice in receptor solubilization buffer. Aliquots (50 μL) of the beads were pelleted in microfuge tubes and incubated with the indicated amounts of either ammonium sulfate precipitated culture media or ascites fluid for 1 h at room temperature. The beads were then washed 3 times and incubated with 20–50 fmol of solubilized receptor overnight in the cold with constant mixing. After centrifugation, the amount of receptor remaining in the supernatant was determined by the binding of [^{3}H]QNB, using the ammonium sulfate precipitation method of Hurko (1978).

Immunoblot Analysis. SDS–polyacrylamide gel electrophoresis was performed by using the discontinuous system of Laemmli (1970) with a 9% polyacrylamide separation gel. The proteins were either stained with silver according to the method of Wray et al. (1981) or were electrophoretically transferred to nitrocellulose by the method of Towbin et al. (1979). The nitrocellulose was incubated with 20 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, 10% bovine hemoglobin, 5% horse serum, and 0.25% gelatin for 1 h at room temperature. The nitrocellulose was then incubated with ammonium sulfate precipitated medium from various hybridoma cell lines diluted in antibody buffer (20 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, 5% horse serum, 0.5% Tween 20, and 0.25% gelatin) for 12–20 h at room temperature. Following several rinses in antibody buffer, the nitrocellulose was incubated for 2 h at room temperature with alkaline phosphatase conjugated goat anti-mouse Ig(G,A,M) diluted in antibody buffer. The nitrocellulose was then rinsed, and the bound antibodies were visualized as described by Smith and Fisher (1984) by incubation of the nitrocellulose in 50 mM sodium glycinate, pH 9.6, 0.1 mg/mL *p*-nitrotetrazolium blue chloride, 0.05 mg/mL 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, 4 mM $MgCl_2$, and 0.5% Tween 20. The reaction was stopped by rinsing with 20 mM NaH_2PO_4, pH 7.4, 150 mM NaCl, and 0.5% Tween 20.

Results and Discussion

Over a dozen fusions were carried out by using a variety of in vivo and in vitro immunization procedures in an attempt to prepare specific anti-mAChR antibodies. The unsuccessful attempts included fusions performed after in vivo subcutaneous immunization with purified mAChR emulsified in Freund's adjuvant and in vitro immunization of lymphocytes with purified receptor, as well as in vivo immunization with anti-ligand mAbs (Gainer & Nathanson, 1986) in an attempt to isolate anti-idiotype antibodies. All of the mAbs described here resulted from a single fusion using the spleen of a mouse immunized intraperitoneally with the purified receptor emulsified in a mixture of monphosphoryl lipid A and trehalose dimycolate (see Materials and Methods). The hybridoma cells were first tested by ELISA for immunoreactivity using a crude

Table I: Properties of Monoclonal Antibodies[a]

clone	subtype	immunoblot	immunoprecipitation
31-1C10	IgG_1	++	+
31-1D1	IgG_1	++	+
31-3B7	IgG_1	++*	+
31-4B9	IgG_1	±	±
31-4E5	IgM	±	–
31-4E7	IgG_1	±	±
31-5B5	IgG_1	+	+
31-5G2	IgM	+	±
31-10D2	IgG_1	++*	+

[a] The subtype of the mAbs was determined by Ouchterlony immunodiffusion as described under Materials and Methods. Immunoprecipitation of the solubilized porcine mAChR was tested as described in Figure 3A; 300 μL of antibody-containing medium was used (symbols: +, >70% of the receptor was precipitated; ±, 15–35% of the receptor was precipitated; –, <10% of receptor was precipitated); each clone was tested in at least two independent experiments in duplicate. Immunoblot analysis was carried out as described in Figure 2 (symbols: ++, reacted strongly; +, reacted moderately; ±, reacted barely visibly; *, reacted both with the main mAChR band and with 32000-kilodalton polypeptide).

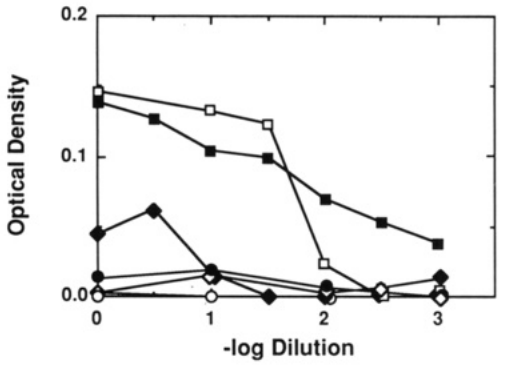

FIGURE 1: ELISA analysis of reaction of mAbs with mAChR. Increasing dilutions of the anti-mAChR mAbs 31-1D1 (■), 31-1C10 (◆), 31-3B7 (□) and the anti-PrBCM mAb 12-2G7 (◇) were tested on highly purified (>95% theoretical specific activity) mAChR, and 31-1D1 (●) and 31-3B7 (○) were tested on the nonreceptor-containing atrial glycoprotein preparation as described under Materials and Methods.

preparation of porcine atrial mAChR (5–10% theoretical specific activity). Of 338 hybridomas tested, 53 reacted with the crude receptor preparation. Of these, 14 also reacted with highly purified preparations of the mAChR (>95% theoretical specific activity). Nine of ten that were selected for further characterization were shown to be specific for the mAChR. The properties of these nine monoclonal antibodies are summarized in Table I.

Hybridoma clones were first tested by ELISA for the ability to specifically recognize the mAChR. Representative ELISA assays are shown in Figure 1. The monoclonal antibodies reacted strongly with the purified mAChR but not with a glycoprotein fraction from pig atria that did not contain any mAChR binding activity. No reactivity in the ELISA was seen when mAbs 12-2G7 (Figure 1) and 12-5B11 (data not shown), which recognize the muscarinic alkylating antagonist PrBCM but not the mAChR (Gainer & Nathanson, 1986; Norman & Nathanson, 1987), were tested on the purified receptor. The reaction of the mAbs with the purified receptor preparation but not with the nonreceptor-containing porcine atrial glycoprotein preparation suggests that the antibodies had a high degree of specificity for the mAChR.

To confirm the specificity of the mAbs for the mAChR, the antibodies were subjected to immunoblot analysis against

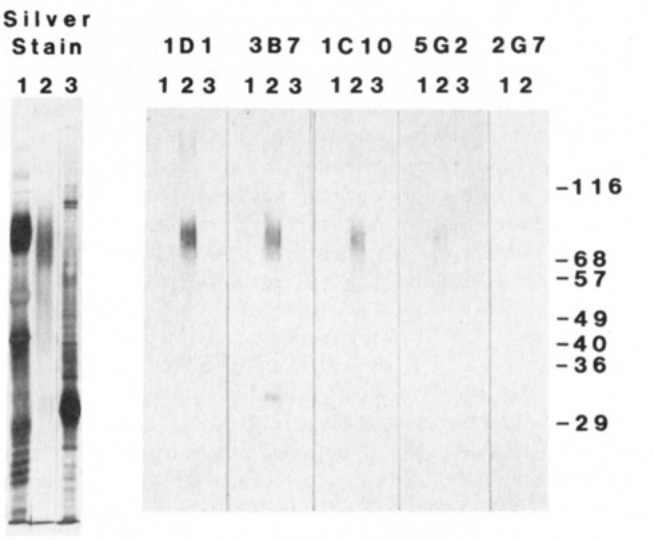

FIGURE 2: Immunoblot analysis of anti-mAChR monoclonal antibodies. Samples were subjected to SDS–polyacylamide gel electrophoresis and either silver stained or transfered to nitrocellulose as described under Materials and Methods. The nitrocellulose was probed with the anti-mAChR mAbs 31-1D1, 31-3B7, 31-1C10, and 31-5G2, as well as with the anti-PrBCM mAb 12-2G7. Silver-stained gel: lane 1, 3 μg of nonreceptor-containing pig atrial glycoprotein preparation; lane 2, 168 ng of purified mAChR; lane 3, 1.2 μg of urea-stripped rod outer segment membranes. Nitrocellulose immunoblot: lane 1, 25 μg of nonreceptor-containing pig atrial glycoprotein preparation; lane 2, 325 ng of purified mAChR; lane 3, 3 μg of urea-stripped rod outer segment membranes.

highly purified preparations of receptor, against the nonreceptor-containing porcine atrial glycoprotein preparation, and against rod outer segment membranes. The results are summarized in Table I, and representative immunoblots are shown in Figure 2. As described previously (Peterson et al., 1984, 1986), the purified receptor appears as a broad band of approximately 60000–75000 daltons on an SDS gel due to extensive glycosylation (28% by weight). As seen in Figure 2, the glycoprotein preparation from pig atria not containing any mAChR binding activity contains a major component that comigrates with a mobility similar to that of the mAChR. As an additional control for specificity, the antibodies were also tested for reactivity with rod outer segment membranes (a generous gift of Dr. Gary Johnson, University of Massachusetts Medical School), which contain large amounts of rhodopsin, a protein of molecular weight approximately 40000, which molecular cloning has indicated is structurally homologous to the mAChR (Kubo et al., 1986b; Peralta et al., 1987). None of the antibodies reacted with any of the polypeptides present in the pig atrial glycoprotein or rod outer segment membrane preparations. Four of the mAbs reacted very strongly with the purified receptor, two reacted moderately, and three exhibited reactivity that was rather weak but detectable. Two of the strongly reacting antibodies recognized an epitope that was also present on a polypeptide of 32000 daltons, which we assume is a proteolytic fragment derived from the muscarinic receptor. The original polyclonal serum from the immunized mouse whose spleen was used to produce these hybridomas also reacted with both the main purified receptor band as well as the 32000 band (data not shown). The monoclonal antibodies also reacted with some high molecular weight material in the receptor preparations. Because this high molecular weight immunoreactive material represents a relatively minor fraction of the total immunoreactivity in fresh preparations of purified mAChR and becomes the major component after storage for several weeks at 0 °C (data not

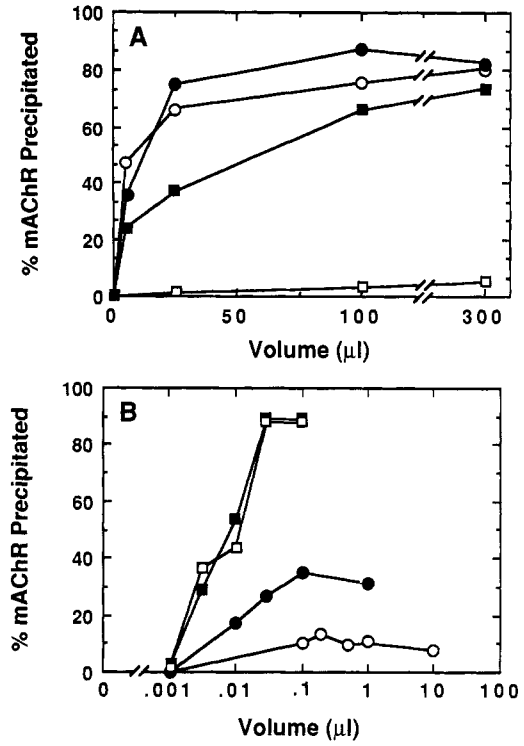

FIGURE 3: Immunoprecipitation of solubilized mAChR. (A) Increasing concentrations of hybridoma culture medium from the anti-mAChR mAbs 31-1D1 (■), 31-1C10 (●), and 31-3B7 (○) and the anti-PrBCM mAb 12-2G7B (□) were tested for the ability to precipitate porcine cardiac mAChR as described under Materials and Methods. (B) Increasing concentrations of mAb 31-1D1 ascites fluid were tested for the ability to immunoprecipitate the mAChR from pig heart (□), rat heart (■), rat cortex (○), and rat cerebellum (●) as described under Materials and Methods. Note that approximately 1000-fold less ascites fluid is required to precipitate the pig heart mAChR compared to hybridoma culture medium.

shown), it is likely that this represents aggregated receptor. The control anti-PrBCM mAbs did not show any reactivity with the purified mAChR. The results of the immunoblot analysis indicate that these antibodies have a high degree of specificity for the muscarinic receptor.

The ability of the mAbs to recognize the mAChR from pig heart in solution after detergent solubilization was tested by immunoprecipitation. As summarized in Table I, four of the mAbs were able to precipitate >70% of the pig atrial mACR (Figure 3), five were able to precipitate a small but significant fraction of the receptor, and one exhibited no detectable precipitation. No receptor was precipitated with the anti-PrBCM mAb 12-2G7 (Figure 3A) or 12-5B11 (data not shown). Thus, the mAbs are able to recognize and immunoprecipitate the detergent-solubilized porcine atrial mAChR.

Because different regions of the central nervous system contain different subtypes of mAChR, the ability of mAb 31-1D1 to immunoprecipitate the mAChR solubilized from various brain regions was determined. Rat tissue was used for these studies because the most detailed data on the distribution of ligand binding subtypes in the brain have been obtained in this species (Giraldo et al., 1987) and because sufficient amounts of freshly dissected tissue could be obtained relatively easily. To ensure that the mAbs which were raised against the purified porcine cardiac receptor could also recognize the receptor from rat, the ability of the antibodies to immunoprecipitate rat cardiac mAChR was determined. Similar concentrations of antibody 31-1D1 were required to precipitate the receptor from pig heart and rat heart, with >85% of both the porcine and rat cardiac receptors precipitated (Figure 3B). Antibodies 31-1C10 and 31-3B7 also were able to precipitate the rat heart mAChR (data not shown).

In contrast to the results seen with porcine and rat heart mAChR, antibody 31-1D1 was able to maximally precipitate only approximately one-third of the rat cerebellar receptor (Figure 3B). Antibodies 31-1C10 and 31-3B7 also precipitated only a portion of the receptor from rat cerebellum. Ligand binding studies have shown that the mAChR in rat cerebellum exhibits a single class of low-affinity sites for pirenzepine with 90% of the sites having a high affinity for AF-DX 116 and thus is primarily of the cardiac M2 subtype (Giraldo et al., 1987). The precipitation by 31-1D1, 31-1C10, and 31-3B7 of only a portion of the receptor from rat cerebellum suggests that the cerebellar receptor may be heterogeneous with respect to the epitopes recognized by the mAbs. This heterogeneity could arise from structural differences due to partial degradation of the receptor, from differences in primary amino acid sequence, or from differences in covalent modifications.

Concentrations of antibody 31-1D1, which were 300-fold higher than that required to precipitate the mAChR solubilized from rat heart, were ineffective in precipitating significant quantities of mAChR from cerebral cortex (Figure 3B). Antibodies 31-1C10 and 31-3B7 also were unable to precipitate the mAChR solubilized from rat cortex (data not shown). The mAChR in the cortex has been shown by Northern blot analysis and radioligand binding experiments to consist predominantly of the M1 and glandular M2 subtypes and little if any of the cardiac M2 subtype (Kubo et al., 1986b; Giraldo et al., 1987). These immunoprecipitation results therefore demonstrate that 31-1D1, 31-1C10, and 31-3B7 are specific for the cardiac M2 form of the muscarinic receptor.

In conclusion, we have isolated a series of monoclonal antibodies raised against the highly purified porcine cardiac muscarinic acetylcholine receptor. These mAbs were shown by ELISA and by immunoblot analysis to be specific for the mAChR by their ability to recognize the receptor but not other glycoproteins in the pig heart. Several of these antibodies are able to quantitatively precipitate the mAChR solubilized from pig and rat heart, but only a portion of the mAChR from cerebellum and none of the receptor from cerebral cortex. These results thus indicate that these antibodies are specific for the cardiac form of the mammalian mAChR. These antibodies should prove useful in further studies on the regulation, function, and structure of the mAChR.

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A Pentapeptide from the Laminin B1 Chain Mediates Cell Adhesion and Binds the 67 000 Laminin Receptor

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ABSTRACT: Laminin promotes epithelial cell adhesion in part through a site of nine amino acids CDPGYIGSR on the B1 chain. Using smaller synthetic peptides from this sequence as well as various peptides with amino acid substitutions, we find that the minimum sequence necessary for efficient cell adhesion as well as receptor binding is YIGSR. The deletion of tyrosine or the substitution of arginine in the peptides resulted in a significant loss of activity. The presence of an amide group on the terminal arginine of either peptide increases activity significantly. YIGSR is active in promoting the adhesion of a variety of epithelial cells; however, it is inactive with chondrocytes, fibroblasts, and osteoblasts.

Laminin, the major noncollagenous glycoprotein in basement membranes (Timpl et al., 1979; Chung et al., 1979), has various biological activities (Kleinman et al., 1985; Timpl & Dziadek, 1986) including promoting epithelial cell adhesion via a 67-kilodalton (kDa) cell surface receptor (Rao et al., 1982; Malinoff & Wicha, 1983; Lesot et al., 1983). Laminin is composed of three chains designated A (M_r 400 000), B1 (M_r 210 000), and B2 (M_r 200 000) that are arranged in a cruciform-like structure (Engel et al., 1981). The laminin chains have been cloned (Barlow et al., 1984; Sasaki et al., 1987), and the B1 (Sasaki et al., 1987) and B2 chains as well as much of the A chain have been sequenced (Sasaki, unpublished results). Seven distinct structural domains occur along the B1 chain, and the B2 chain has a homologous but not identical structure. Recently, using synthetic peptides of approximately 20 amino acids corresponding to sequences in these domains and antibodies prepared to the peptides, we found that an antibody to a peptide from a region near the intersection of the three chains in domain III inhibited cell attachment to laminin while the peptide itself was inactive (Graf et al., 1987). Various peptides from adjacent sequences were synthesized and a nine amino acid peptide, CDPGYIGSR, in the B1 chain was found to be active in cell adhesion, in stimulating cell migration, and in cell surface receptor binding. Here we test smaller peptides contained within this sequence as well as various amino acid substitutions in the active peptide to determine the minimum sequence required for cell attachment and for receptor binding. Our studies