N-Cadherin Is Stably Associated with and Is an Acceptor for a Cell Surface N-Acetylgalactosaminylphosphotransferase*

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Calcium-dependent cell-cell adhesion among embryonic chick neural retina cells is mediated by N-cadherin, a 130,000-Da integral membrane protein. We have reported that the ability of chick neural retina cells to form calcium-dependent cell-cell adhesion is also correlated with the presence of an N-acetylgalactosaminyltransferase at the cell surface (Balsamo, J., and Lilien, J. (1982) J. Biol. Chem. 257, 349-354). This enzyme transfers N-acetylgalactosaminylphosphate to endogenous acceptors (Balsamo, J., Pratt, R. S., and Lilien, J. (1986) Biochemistry 25, 5402-5407) and is tightly associated with these acceptors forming a complex which can be isolated from neutral detergent extracts of plasma membranes. We now report that N-cadherin is present in the complex and is an acceptor for the N-acetylgalactosaminylphosphotransferase. Antibodies prepared against N-cadherin precipitate the transferase containing complexes from neutral detergent extracts; however, when the complexes are dissociated by treatment with sodium dodecyl sulfate prior to immunoprecipitation, only N-cadherin is precipitated. Similarly, anti-transferase antibodies immunoprecipitate N-cadherin containing complexes prior to disruption of the complex with sodium dodecyl sulfate. Catalysis of the transferase reaction in the complex results in N-cadherin molecules containing terminal N-acetylgalactosamine linked to an oligosaccharide chain via a phosphodiester bond.

Calcium-dependent adhesive components comprise a family of closely related yet immunologically distinct molecules (Shirayoshi et al., 1986; Crittenden et al., 1987). One such molecule, N-cadherin, is localized predominantly to neural tissues (Hatta and Takeichi, 1986; Crittenden et al., 1987). It was originally identified on embryonic chick neural retina as a cell surface glycoprotein with a molecular mass of approximately 130,000 Da and a pl of 4.8, and its presence was shown to correlate with the ability of cells to form calcium-dependent cell-cell adhesions (Cook and Lilien, 1982; Grunwald et al., 1982). N-cadherin was subsequently shown to inhibit cell-cell adhesion among embryonic chick neural retina cells (Crittenden et al., 1987) and embryonic mouse brain cells (Hatta et al., 1986). In vivo N-cadherin may function in the nervous system to modulate or mediate cell-cell interactions. As a first step in defining the biochemical and biological relationship between N-cadherin and the transferase, we report that N-cadherin is indeed an acceptor for the enzyme and is part of the multimolecular transferase-acceptor complex.

Correlations between the presence of the GalNACP-transferase and N-cadherin at the cell surface suggested the possibility that the two might interact as enzyme and acceptor to modulate or mediate cell-cell interactions. As a first step in defining the biochemical and biological relationship between N-cadherin and the transferase, we report that N-cadherin is indeed an acceptor for the enzyme and is part of the multimolecular transferase-acceptor complex.

MATERIALS AND METHODS

Antibodies. RR1 is a polyclonal rabbit anti-N-cadherin antibody specific to neural tissues (Crittenden et al., 1987). This antibody was affinity-purified on nitrocellulose replicas of N-cadherin (Smith and...
Iodination of Cultured Cells—An average of 20 neural retinas were dissected from 9-day-old embryos and dissociated with trypsin as described (Geller and Lilien, 1983). Cells were plated in 10-cm Petri dishes at a density of ~10^6 cells/6 ml of HBSGK (20 mm Hepes, 150 mm NaCl, 2 mm glucose, 3 mm KCl, pH 7.4) containing 1 mm Ca^2+ and 0.5% gentamycin (Sigma). After an incubation period of 24 h at 37 °C as still cultures, the cells were harvested with a Pasteur pipette, washed twice in HBSGK, and labeled by lactoperoxidase-catalyzed iodination (Geller and Lilien, 1983).

Preparation of GalNAc-transferase-Acceptor Complexes from Iodinated Cells and Immunoprecipitation with Antibodies—The iodinated cells were lysed in 1 ml of HSTI. Particles containing transferase-acceptor, called H particles, were prepared as described previously (Balsamo et al., 1986a). Aliquots of equal volume were incubated with control rabbit IgG, Cl, or 1B11 overnight at 4 °C. Aliquots were treated for fluorography with Amplify (Amersham Corp.) and exposed to x-ray film for 2 weeks at -70 °C.

Immunoblot Analysis of Immunoprecipitated H Particles—Aliquots of 100-μl H particles (100-200 μg of protein) were incubated with 10 μl of ascites fluid containing 6H5 IgG or an unrelated mouse IgG (Balsamo et al., 1986a). After 1 h on ice, 100 μl of a 20% suspension of protein A-Sepharose which had previously been incubated with rabbit-anti-mouse IgG was added and the mixture was incubated for another hour on ice. The protein A-Sepharose pellet was washed 3 times with HST (20 mm Hepes, pH 7.5, 150 mm NaCl, 1% Triton X-100), and the antigen-antibody complex was eluted with SDS sample buffer, fractionated on a 7% SDS-polyacrylamide gel, and transferred to nitrocellulose. The protein blots were then incubated with 1:500 RR1 serum as described above.

Immunoprecipitation of GalNAc-transferase Activity from H Particles by Anti-N-cadherin Antibodies—Aliquots of H particle preparations (100–200 μg of protein) were incubated with a 1:100 dilution of RR1 or control IgG for 1 h on ice. Protein A-Sepharose in HSTI was then added and the mixture incubated for another 30 min. The protein A-Sepharose pellet was washed several times with HSTI.

In order to measure endogenous transferase activity, the final pellet was resuspended in 100 μl of HSTI and incubated with 2 μl of [3H]UDP-GalNAc (Du Pont-New England Nuclear; 100 μCi/ml; 8.7 Ci/mmol) for 60 min at 37 °C under continuous agitation. Radioactivity incorporated into macromolecules was recovered and measured as described previously (Balsamo et al., 1986a). Alternatively, aliquots of H particle preparations were applied to columns containing NCD-2 or Cl IgG coupled to Affi-Gel 10 (Bio-Rad; 2 mg of IgG/0.5 ml of gel). The columns were washed with 20 volumes of HST followed by 1 volume of 10 mM diethylamine, 1% Triton X-100, pH 10.0. The bound material was eluted with 2 volume washes of 10 mM diethylamine, 1% Triton X-100, pH 11.5. After overnight dialysis against HST, aliquots of the eluted material were assayed for transferase activity as described above.

Preparation of 32P- and 3H-Labeled GalNAc-transferase Products and Immunoprecipitation by Anti-N-cadherin Antibodies—[32P]UDP-GalNAc was prepared in our laboratory essentially as previously described (Balsamo et al., 1986b) using [32P]ATP and galactokinase prepared from galactose-adapted yeast (Carlson and Rosenman, 1972). In a typical preparation, H particles from 60 retinas were incubated in 2 ml of HSTI, ~50% sucrose, 2 mm MnCl₂, and 50 μl of [32P]UDP-GalNAc (2 x 10⁵ cpm) (Amersham Corp.; 61 mCi/mmol; 35 mCi/ml). After centrifugation at 100,000 × g for 30 min, the supernatant fluid was divided into aliquots of equal volume and incubated either with RR1, Cl, or preimmune rabbit IgG, overnight at 4 °C. The antigen-antibody complex was recovered, washed as described above, eluted, and analyzed by SDS-PAGE. The 32P-labeled gels were exposed to x-ray film for 1 week at -70 °C in the presence of enhancing screens (Cronex); the 3H-labeled gels were treated for fluorography with Amplify (Amersham Corp.) and exposed to x-ray film for 2 weeks at -70 °C.

Phosphodiesterase Labile Linkages in N-Cadherin Labeled in Cultured Cells—Single cells were prepared from neural retinas by standard trypsin treatment (Grunwald et al., 1980). Cells from 20-9 day-old neural retinas were cultured overnight as monolayers in 25 ml of HBSGK containing 1 mm Ca²⁺, 0.5% gentamycin, and 1 μCi of [3H] orthophosphate (Du Pont-New England Nuclear). After an incubation period of 24 h at 37 °C as still cultures, the cells were harvested with a Pasteur pipette, washed twice in HBSGK, and labeled by lactoperoxidase-catalyzed iodination (Geller and Lilien, 1983).
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Du Pont-New England Nuclear, 29 mCi/mmol; 1 mCi/ml) at a concentration of 10^5 cells/ml. The cells were harvested, washed, and homogenized in HSTI. After 60 min on ice, the insoluble residue was pelleted and the supernatant fluid heated with 1% SDS. After dilution of the SDS to 0.2%, the solution was incubated with RRI overnight at 4°C under gentle agitation. The immunoprecipitates were bound to protein A-Sepharose, and the resulting pellets were treated in different ways, depending on the isotope used. ^32P-Labeled N-cadherin was recovered following SDS-PAGE. The gel slabs were soaked in two changes of 10% glycerol, at room temperature, under gentle agitation and dried under vacuum at 80°C between two sheets of cellophane which had been treated as described previously (Cook and Lilien, 1982). The ^32P-labeled bands corresponding to N-cadherin were excised from dried polyacrylamide gels using the autoradiograph as a guide. The macromolecules were eluted from the gel slices in Tris-glycine buffer containing 0.1% SDS using a Bio-Rad mini-eluter.

To determine if N-cadherin contained ^32P in phosphodiesterase-labile linkages, the eluted N-cadherin was divided into aliquots of equal counts and incubated with 2 mM phenylmethylsulfonyl fluoride, 20 mM EDTA, and with or without 5 units/ml bovine spleen phosphodiesterase (Sigma). After 2 h at 37°C, an equal volume of 2 × SDS sample buffer was added, and the reaction mix was boiled for 3 min and loaded onto a 7% polyacrylamide gel. The migration of the labeled N-cadherin was determined by autoradiography.

To examine the nature of the terminal phosphodiester-linked sugar, equal aliquots of the ^3H-labeled antigen-antibody complexes precipitated by protein A-Sepharose were resuspended in 20 mM Tris, pH 7.8, containing 150 mM NaCl, 15 mM Mg++, 50 μg/ml antipain and incubated with or without 0.5 units of phosphodiesterase (Sigma, snake venom) for 1 h with constant mixing. The released labeled material was analyzed by chromatography on Whatmann no. 3MM paper using 95% ethanol, 1 M ammonium acetate, pH 3.8 (52). ‘H-Labeled GalNAcP used as a marker in paper chromatography was produced by treating [3H]UDP-GalNAc with the snake venom phosphodiesterase. Unlabeled GalNAcP was used to determine the migration of the free GalNAc monosaccharide and was detected with ninhydrin (1% in acetone). The position of the radioactive peaks was determined by slicing the appropriate lanes into 1-cm pieces and counting by liquid scintillation.

RESULTS

N-Cadherin Is Associated with an N-Acetylgalactosamine-phosphotransferase in a Stable Particulate Complex—Analysis of two-dimensional gels of adhesion-competent retina cells, surface-labeled via lactoperoxidase-catalyzed iodination, reveals a selected population of iodinated polypeptides (Geller and Lilien, 1983). The polypeptide corresponding to a molecular mass of approximately 220,000 Da and a pI of 5.0 is the GalNAcP-transferase, as identified by monoclonal antibodies (Balsamo et al., 1986a); N-cadherin migrates to a position corresponding to a molecular mass of approximately 130,000 Da and a pI of 5.0 (Crittenden et al., 1987). Neutral detergent-insoluble complexes enriched for endogenous GalNAcP-transferase activity prepared from the ^125I-labeled cells contain both labeled N-cadherin and transferase (Fig. 2A). These cell surface-associated GalNAcP-transferase-acceptor complexes...
were originally termed H particles because of their behavior on sucrose gradients; we will continue to use this terminology.

Antibodies directed against N-cadherin and the transferase were used to demonstrate the presence of both molecules in a single complex. Immunoprecipitates of 125I-labeled H particles with the anti-N-cadherin antibody, C1, are enriched for N-cadherin, its fragment, gp70 (Crittenden et al., 1987), and the GalNAcP-transferase along with as yet unidentified components (compare Fig. 2, A and C). In contrast, when H particles are dissociated by heating in 1% SDS prior to incubation with the anti-N-cadherin antibodies, only N-cadherin and gp70 are precipitated (Fig. 2E).

The complementary approach using a monoclonal antibody directed against the GalNAcP-transferase to immunoprecipitate intact iodinated H particles results in a two-dimensional gel profile identical to the one obtained using C1 as the immune reagent (Fig. 2B). No immunoprecipitation of N-cadherin or transferase is detected when control IgG is substituted for C1 or 1B11 either prior to (Fig. 2D) or following SDS treatment (not shown). Immunoprecipitation of both N-cadherin and the transferase with either anti-N-cadherin or anti-transferase antibodies from iodinated H particles prior to dissociation by SDS indicates that these components are stably associated. The stability of the association is also reflected in the consistent array of components, in addition to the transferase and N-cadherin, immunoprecipitated by each of the antibodies. The same results were obtained using another experimental approach and a different set of antibodies. Unlabeled intact H particles were immunoprecipitated with the anti-transferase antibody 6H5. The precipitate was analyzed by SDS-PAGE and immunoblotted with RR1. The resulting immunoblots show that N-cadherin is present in the same complex as the transferase (Fig. 3).

Anti-N-cadherin antibodies are also effective in immunoprecipitating the endogenous GalNAcP-transferase activity from intact H particles. As shown in Fig. 4, transferase activity can be specifically immunoprecipitated from H particles by RR1 (panel A) and specifically bound to NCD-2 or C1 covalently coupled to Affi-Gel 10 (panel B). The antibodies do not recognize the enzyme itself; no activity is recovered in the immunoprecipitate when soluble transferase, released from particles, is incubated with the anti-N-cadherin antibody RR1 and assayed with an exogenously added acceptor (results not shown) (Balsamo et al., 1986a).

**In Situ Labeled N-Cadherin Contains GalNAc Phosphate—** To eliminate the possibility that glycosylation of N-cadherin by the transferase is a result of an artificial association caused by the Triton extraction, we examined in situ labeled

**FIG. 3. Western blot analysis of N-cadherin in H particles.** Preparations of H particles were subjected to electrophoresis on polyacrylamide-SDS gels either before or after immunoprecipitation with anti-transferase antibody, 6H5. Nitrocellulose replicas of SDS-PAGE were reacted with RR1 as described under "Materials and Methods." Lane a, intact H particles; lane b, H particles immunoprecipitated with 6H5; lane c, control mouse IgG. Numbers to the left of figure represent the migration of prestained molecular weight standards \( \times 10^{-3} \). The arrow indicates the position of N-cadherin.
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**FIG. 5.** Electrophoretic analysis of H particle-glycosylated acceptors immunoprecipitated by anti-N-cadherin antibodies. Panel A, immunoprecipitation of ¹⁴C-labeled glycosylated H particle acceptors: lane a, total; lane b, RR1. Panel B, immunoprecipitation of ³²P-labeled acceptors: lane a, total; lane b, RR1; lane c, C1; lane d, control serum. Panels A and B represent two different SDS-PAGE systems. Numbers on the left of the figure represent molecular weight standards \( \times 10^{-3} \). The arrow indicates the position of N-cadherin.

N-cadherin for the presence of phosphodiesterase-labile GalNAc residues. First, intact cells were shown to incorporate [³²P]orthophosphate into oligosaccharide chains in N-cadherin. Immunoprecipitated N-cadherin eluted from SDS-polyacrylamide gels were treated with bovine spleen phosphodiesterase in the presence of protease inhibitors and EDTA, which is necessary for the phosphodiesterase activity but inhibits alkaline phosphatase. The enzyme-treated material shows a dramatic reduction in the density of label at 130,000 Da as well as a new band of radioactivity migrating with the dye front on SDS gel electrophoresis (Fig. 6). This reduction is not due to protein degradation, as evidenced by the amount of C1-reactive material in the control and phosphodiesterase-treated samples (Fig. 6, lower panel).

Finally, N-cadherin immunoprecipitated from cells labeled in culture with [³H]glucosamine was digested with phosphodiesterase, and the released sugar residues were identified by paper chromatography. [³H]GalNAcP generated by digestion of [³H]UDP-GalNAc with snake venom phosphodiesterase was used as a marker for the migration of GalNAcP. As can be seen in Fig. 7, the radiolabeled sugar resulting from phosphodiesterase digestion of N-cadherin comigrates with authentic GalNAcP. Radioactivity comigrating with unphosphorylated GalNAc is also seen, probably due to the lability of the terminal phosphate. All radioactivity remains at the origin of the chromatogram of control samples treated under similar conditions but without the enzyme.

**DISCUSSION**

The data presented here demonstrate that the neural calcium-dependent cell adhesion molecule, N-cadherin, is intimately associated with, and is an acceptor for, the neural retina cell surface GalNAcP-transferase. Using antibodies directed against N-cadherin, both N-cadherin and the associated transferase molecule were immunoprecipitated from intact transferase-acceptor complexes. The complementary approach, using an antibody prepared against the GalNAcP-transferase, also resulted in coprecipitation of the transferase and N-cadherin. The coprecipitation of the two molecules is
not a result of immunological cross-reactivity; when the association among the particulate transferase-acceptor components is disrupted by treatment with ionic detergent, only N-cadherin is immunoprecipitated by the anti-N-cadherin antibody. Also, solubilized GalNAcP-transferase released from particles on SDS treatment is not recognized by anti-N-cadherin antibodies.

The complex contains components in addition to the transfase and N-cadherin. Other iodinated cell surface components are consistently present in the immunoprecipitated complex independent of the antibody used. This implies that the complex reflects an in situ association (see below). Additional components, not accessible to iodination either because of their surface orientation or their presence at the cytoplasmic side of the plasma membrane, may also be present in the complex. It has been reported that cadherins are associated with the cytoskeleton (Hirano et al., 1987) suggesting the possibility that the complex may contain cytoskeletal elements.

N-Cadherin in the particulate complex acts as an acceptor for the transfase. Preparations of GalNAcP-transferase-acceptor complexes catalyze the transfer of GalNAcP into either the sugar or the phosphate residue from UDP-GalNAc to N-cadherin. Other unidentified components in the complex are also glycosylated but not recognized by the anti-N-cadherin antibodies. It is not known if these other glycosylated components are represented in the two-dimensional gels of iodinated particles or whether their glycosylation is representative of an in situ reaction.

The transfer of GalNAcP to N-cadherin does occur in situ and is therefore not a result of an artificial association of the two molecules. Intact cells incorporate $^{32}$Porthophosphate into oligosaccharides of N-cadherin in phoshodiester-containing linkages. Furthermore, $^{3H}$glucosamine incorporated into N-cadherin by intact cells can be released as GalNAcP by digestion of immunoprecipitated N-cadherin with phoshodiesterase.

We have previously pointed out correlations between the presence of the transfase at the cell surface and the ability of cells to form calcium dependent cell cell adhesions (Balsamo and Lilien, 1982; Balsamo et al., 1986a). Whether cells are prepared with an intact calcium-dependent adhesive system or whether they must undergo repair processes to reconstitute the system, the transfase is invariably associated with adhesive competence. Our present results go beyond these correlative findings to show a physical association between the calcium-dependent adhesive molecule, N-cadherin, and the enzyme. Furthermore, N-cadherin is an acceptor for the transfase.

The transfase and N-cadherin may be physically associated in other tissue systems. We have reported that the transfase is present at the vertebrate neuromuscular junction (Scott et al., 1989), and more recently we have found that N-cadherin is also present at the neuromuscular junction. Furthermore, both of these components are coordinately regulated by innervation. At present we can only speculate on the physiological role that the transfase plays. The transfase may play the role of a receptor for N-cadherin, actually participating in the formation of cell-cell adhesions in a manner reminiscent of the hypotheses put forth by Roseman (1970). A galactosyltransferase has been shown to act in just this capacity in the interaction of mouse sperm and eggs (Hathaway and Shur, 1988). Another possibility is that the GalNAcP-transferase may play a more indirect role by modulating the formation or turnover of adhesive bonds via glycosylation of N-cadherin. Additionally, like phosphodiester-linked terminal sugars in other systems (von Figura and Hasilik, 1986; Marchase, 1987), terminal glycosylation of N-cadherin may be involved in targeting the molecule to a specific cellular location, possibly as a prerequisite to adhesion. We are at present pursuing these possibilities.

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