Differential Phosphorylation of the Gap Junction Protein Connexin43 in Junctional Communication-competent and -deficient Cell Lines

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Abstract. Connexin43 is a member of the highly homologous connexin family of gap junction proteins. We have studied how connexin monomers are assembled into functional gap junction plaques by examining the biosynthesis of connexin43 in cell types that differ greatly in their ability to form functional gap junctions. Using a combination of metabolic radiolabeling and immunoprecipitation, we have shown that connexin43 is synthesized in gap junctional communication-competent cells as a 42-kD protein that is efficiently converted to a ~46-kD species (connexin43-P_2) by the posttranslational addition of phosphate. Surprisingly, certain cell lines severely deficient in gap junctional communication and known cell–cell adhesion molecules (S180 and L929 cells) also expressed 42-kD connexin43. Connexin43 in these communication-deficient cell lines was not, however, phosphorylated to the P_2 form. Conversion of S180 cells to a communication-competent phenotype by transfection with a cDNA encoding the cell–cell adhesion molecule L-CAM induced phosphorylation of connexin43 to the P_2 form; conversely, blocking junctional communication in ordinarily communication-competent cells inhibited connexin43-P_2 formation. Immunohistochemical localization studies indicated that only communication-competent cells accumulated connexin43 in visible gap junction plaques. Together, these results establish a strong correlation between the ability of cells to process connexin43 to the P_2 form and to produce functional gap junctions. Connexin43 phosphorylation may therefore play a functional role in gap junction assembly and/or activity.

Gap junctions are composed of transmembrane channels that directly link the cytoplasms of adjoining cells (Gilula et al., 1972; Bennett and Goodenough, 1978; Loewenstein, 1981). Found in almost all animal tissues, gap junctions are thought to mediate the intercellular transfer of low-molecular weight metabolites and ions. The permeability of the junctional channels can be reversibly altered by a wide variety of effectors, including changes in cytoplasmic pH (Turin and Warner, 1977), free Ca^{2+} (Rose and Loewenstein, 1975), voltage (Spray et al., 1979), cyclic nucleotides (De Mello, 1988; Saez et al., 1986), and transforming viral oncogene activity (Atkinson and Sheridan, 1985) (Azarnia et al., 1988). Gap junction–mediated cell-to-cell communication is clearly important in the function of electrically excitable tissues such as myocardium (De Mello, 1977), smooth muscle (Dewey and Barr, 1962), and nerve (Furshpan and Potter, 1968), and is likely to be critical for maintaining metabolic homeostasis in the avascular lens (Mathias and Rae, 1985; Goodenough et al., 1980). In other cell types, gap junctions have been implicated in embryonic development (Guthrie and Gilula, 1989), cellular differentiation, and growth control (Pitts, 1978; Loewenstein, 1979; Mehta et al., 1986).

Structural studies have demonstrated that the gap junctional channel is composed of a hemichannel (connexon) in the plasma membrane of one cell joined in mirror symmetry with a connexon in the apposing cell membrane (Makowski et al., 1977; Bennett and Goodenough, 1978; Caspar et al., 1988). Each connexon is an oligomer of six protein subunits which delineate an axial aqueous pore. Recent molecular cloning of one of these proteins (Paul, 1986; Kumar and Gilula, 1986) has led to the discovery of a widely distributed family of related gap junction proteins, the connexins (Beyer et al., 1988; Stevenson and Paul, 1989). Connexins are integral membrane proteins that are highly homologous in their extracellular and transmembrane domains but more structurally diverse in their cytoplasmic regions. Evidence that connexins themselves mediate gap junctional communication includes: (*a*) immunocytochemical localization of anticonnexin antibodies to gap junctions but not to nonjunctional plasma membrane (Paul, 1986; Yancey et al., 1989; Beyer et al., 1989; Dermietzel et al., 1989); (*b*) reconstitution of channels after insertion of purified connexins into artificial membranes (Young et al., 1987); (*c*) formation of intercellular low-resistance pathways in paired *Xenopus* oocytes directed by microinjected connexin-encoding mRNAs

(Swenson et al., 1989; Werner et al., 1989); and (*d*) inhibition of dye transfer and electrical coupling by anticonnexin antibodies in several cell types (Fraser et al., 1987; Hertzberg et al., 1985; Yancey et al., 1989; Warner et al., 1984; Traub et al., 1989).

Although much recent work has focused on the cloning and tissue distribution of the various connexins, relatively little is understood about their posttranslational processing and oligomerization. It is not known, for example, how connexins assemble into connexons and subsequently into gap junction plaques, nor what mechanisms regulate these processes. As a first step towards addressing such issues, we recently examined connexin biosynthesis and posttranslational modification in embryonic chick lens, a tissue whose cells are extensively interconnected by gap junctions (Musil et al., 1990). Our studies demonstrated that chick lens epithelial cells synthesize and assemble into gap junctions connexin43, a member of the connexin family first cloned from rat heart (Beyer et al., 1987). Metabolic labeling and immunoprecipitation studies showed that connexin43 is synthesized in lens cells as a ~42-kD protein but is efficiently converted to a slightly slower migrating form within 1–2 h. This posttranslational shift in apparent molecular weight is due entirely to phosphorylation of connexin43, a relevant finding in light of the extensive literature suggesting a role for protein phosphorylation in the regulation of gap junctional communication (Wiener and Loewenstein, 1983; Azarnia et al., 1988; Loewenstein, 1985; Saez et al., 1986).

Phosphorylation can profoundly influence the biological activity, intracellular transport, assembly, or metabolic stability of a protein (Yarden and Ullrich, 1988; Sibley et al., 1987; Cooper and King, 1986). Alternatively, phosphorylation can be the consequence of an opportunistic protein kinase with no obvious effect on the function or fate of a particular substrate. To gain insight into whether posttranslational phosphorylation of connexin43 is of functional significance, we have examined the biosynthesis and intracellular localization of connexin43 in several cell lines that differ markedly in their ability to form functional gap junctions. We report here that junctional communication-competent rat normal rat kidney cells synthesized and posttranslationally processed connexin43 in a manner qualitatively similar to chick lens epithelial cells, phosphorylating it first to a P_1 and then to a slower migrating P_2 form. Mouse S180 cells (Furshpan and Potter, 1968; Mege et al., 1988) and L929 cells (Pitts, 1971; Larson and Sheridan, 1985), which have been reported to be severely deficient in gap junctional communication, also synthesized connexin43 but did not detectably phosphorylate it to the mature P_2 form. Transfection of S180 cells with a cDNA encoding the intercellular adhesion molecule L-CAM[1] corrected both the gap junctional communication (Mege et al., 1988) and connexin43 phosphorylation defects, indicating that lack of specific cell–cell adhesion rather than a mutation in the connexin43 gene was responsible for the parental phenotype. In complementary experiments, ordinarily communication-competent cells treated with known inhibitors of gap junctional permeability no longer detectably processed connexin43 to the P_2 form. Immunohistochemical localization studies indicated that cells expressing the P_2 form of connexin43 invariably accumulated connexin43 in macular regions of the plasma membrane, whereas communication-deficient cell lines showed little or no cell surface anti-connexin43 staining. Together, these results establish a strong correlation between the ability of cells to phosphorylate connexin43 to the P_2 form and to produce functional gap junctions. The lack of junctional communication in L929 and S180 cells is not due to an absence of connexin43 synthesis but may instead be related to a defect in connexin43 phosphorylation, consistent with a functional role for connexin43 phosphorylation in gap junction formation or activity.

1. *Abbreviations used in this paper*: L-CAM, intercellular adhesion molecule; NRK, normal rat kidney.

Materials and Methods

Reagents

Tissue culture reagents were purchased from Gibco Laboratories (Grand Island, NY), except for fetal calf serum which was from Hyclone Laboratories (Logan, UT). Phosphate-free MEM was prepared in the laboratory from tissue culture grade reagents and concentrated MEM vitamin and amino acid solutions. Alpha [^{32}P]-dCTP (>3,000 Ci/mmol) and [^{35}S]methionine (680 Ci/mmol; cell labeling grade) were from New England Nuclear (Boston, MA); $H_3[^{32}P]O_4$ (285 Ci/mg) was from ICN Radiochemicals (Irvine, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Cell Culture

The normal rat kidney (NRK) cell line (kindly provided by Brian Burke, Harvard Medical School, Boston, MA; originally obtained from S. J. Singer, University of California, San Diego, La Jolla, CA) and L929 cell line (purchased from the American Type Culture Collection, Rockville, MD) were grown in DMEM containing 10% FCS, penicillin G, and streptomycin. Mouse sarcoma 180 (S180) cells and S180 cells stably transfected with chicken L-CAM cDNA (S180L cells) were propagated as previously described (Mege et al., 1988). All cell cultures were used on the third day after plating, shortly after attaining confluency. The gap junctional communication competence of the NRK and S180L cell lines was confirmed by dye coupling experiments (not shown); comparable results were obtained when Lucifer yellow was introduced into cells by microinjection (Schuetze and Goodenough, 1982) or by scrape-loading (El-Fouly et al., 1987).

Affinity Purification of Anticonnexin43 Serum

A connexin43-specific synthetic peptide encoding amino acids 252–271 of rat heart connexin43 was used to generate a rabbit anticonnexin43 (252-271) serum as described previously (Beyer et al., 1989). 500 μl of this antiserum was passed through the membrane filter of a MAC-25 cartridge (Memteck Corp., Billerica, MA) that had been conjugated with 5.8 mg of the crude connexin43(252–271) peptide in accordance with the manufacturer's instructions. The bound connexin43-specific antibodies were eluted with 0.1 M glycine at pH 2.2 and immediately neutralized as described by Yancey et al. (1989).

Metabolic Labeling of Cells, Preparation of Cell Lysates, and Immunoprecipitation

Details of the metabolic labeling of cell cultures with [^{35}S]methionine or [^{32}P]orthophosphate are given elsewhere (Musil et al., 1990). At the end of the labeling or chase period, the cultures were solubilized in the presence of 0.6% SDS, boiled, and the resulting cell lysates immunoprecipitated as previously described (Musil et al., 1990).

SDS Gel Electrophoresis and Fluorography

Immunoprecipitated samples were analyzed on SDS-polyacrylamide gels (Laemmli, 1970) as modified by Carr et al. (1987) to maximize electrophoretic separation of proteins in the 40–50-kD molecular weight range. Resolving and stacking gels contained 8% acrylamide/0.32% *N,N*-methylene bisacrylamide and 4% acrylamide/0.16% *N,N*-methylene bisacrylamide, respectively, and electrode buffer consisted of 0.05 M Tris base,

0.38 M glycine, and 0.15% SDS. Gels were processed for fluorography with EN^{3}HANCE (New England Nuclear) using the supplier's suggested protocol and then exposed to prefogged Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY). Quantitative densitometry was conducted with a LKB UltroScan XL laser densitometer (LKB Instruments, Gaithersburg, MD).

Dephosphorylation of Connexin43

Metabolically labeled connexin43 was immunoprecipitated and incubated for 4 h at 37°C in the presence of molecular biology grade calf alkaline phosphatase (Boehringer, Mannheim, FRG) as detailed previously (Musil et al., 1990). For control reactions conducted in the presence of phosphatase inhibitors, 2 mg/ml Na orthovanadate, 10 mM EDTA, and 10 mM PO_4 were added to the phosphatase reaction buffer.

Phosphoamino Acid Analysis of Connexin43

One (NRK cells) or three (S180 cells) confluent 60-mm cell cultures were metabolically labeled for 4 h with [^{32}P]orthophosphate, lysed, and immunoprecipitated with affinity purified anti-connexin43(252-271) antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE and the region of the fixed, dried gel containing ^{32}P-labeled connexin43 excised after autoradiography. Excised gel slices were swelled in 3 ml/gel slice of 30% MeOH, changing the solution once every hour for the first 3 h and then allowing the slices to fix overnight in fresh 30% MeOH. These washes served to elute residual unincorporated [^{32}P] from the sample and facilitated removal of the filter paper onto which the gel had been dried. After an additional 60 min in fresh 30% MeOH, the gel slices were dried by vacuum centrifugation in a Speed Vac Concentrator (Savant Instruments, Hicksville, NY) and rehydrated in 300 μl/gel slice of 50 mM ammonium bicarbonate containing 50 μg/ml TPCK-treated trypsin (Worthington Biochemicals, Freehold, NJ). The samples were incubated at 37°C for 20 h, with the addition of 200 μl/gel slice of fresh trypsin solution after the first 6 h. Gel fragments were removed by centrifugation for 10 min in a microfuge and the supernatant, containing digested connexin43 was transferred to a new tube and dried in a Speed Vac concentrator. After resuspension in 100 μl/sample deionized distilled water and another round of lyophilization, the peptide pellets were hydrolyzed in constant boiling 5.7 N HCl (Pierce Chemical Co., Rockford, IL; 90 μl/sample) at 100°C for 1.5 h. The HCl was removed by four cycles of vacuum centrifugation at room temperature, using 50, 50, and 25 μl of ddH_2O for each successive resuspension of the sample. The final samples were dissolved in 1 μl/sample of dye mix (0.3% each of Xylene cyanol FF, Orange G, and Acid Fuschin) containing nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine internal standards. The samples were spotted onto a 20 × 20 cm cellulose thin-layer chromatography plate (Electron Microscopy Science, Cherry Hill, NJ) and subjected to one-dimensional high-voltage electrophoresis at 1,000 V for 2 h in pyridine/acetate buffer (pH 3.5) as described by Cooper et al. (1983). The positions of ^{32}P-labeled amino acids were determined by autoradiography; the unlabeled internal standards were visualized by ninhydrin staining.

Inhibition of Junctional Permeability with Heptanol

Heptanol (Fisher Scientific, Fair Lawn, NJ) was diluted fresh daily 1:4 in ethanol. 20 μl of this stock solution were added to 10 ml of 37°C DME containing 5% FCS (final concentration of heptanol = 3.5 mM, final concentration of ethanol = 0.15%) and vortexed vigorously for 2 min to ensure that the heptanol was in solution. The medium was then added immediately to cell cultures for the time indicated in the individual experiments. Control experiments demonstrated that 0.15% ethanol alone had no effect on gap junction permeability or connexin43 phosphorylation. Cells chased in the presence of heptanol were lysed as described above except that the rinse and lysis buffers were supplemented with 3.5 mM heptanol.

RNA Isolation and Northern Blots

RNA was isolated by homogenization of tissues or cultured cells in guanidine isothiocyanate followed by centrifugation through CsCl (Chirgwin et al., 1979). RNA samples (10 μg, as estimated from optical absorbance measurements at 260 and 280 nm) were electrophoresed on 1% agarose/formaldehyde gels and blotted onto nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL) by capillary action. RNA was cross-linked to the membrane by exposure to a 300-nm UV transilluminator for 5 min. The blots were hybridized with ^{32}P-labeled probes overnight at high stringency (0.75 M Na_2HPO_4, 5% SDS, 100 μg/ml salmon sperm DNA, pH 7.2, 65°C), and washed three times with 0.3 M Na_2HPO_4 (pH 7.2), 1% SDS, and then twice with 0.015 M Na_2HPO_4, 1% SDS. Washes were for 20 min each at 65°C. Probes were purified by electrophoresis in low melting temperature agarose and radiolabeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase I and hexanucleotide primers as described by Feinberg and Vogelstein (1983). The cDNA probes used were (*a*) a cDNA insert containing the entire coding region of rat heart connexin43 (clone G2; Beyer et al., 1987); (*b*) an 850-bp fragment of rat liver connexin-32 cDNA consisting of the complete protein coding sequence and a small amount of flanking untranslated regions (Swenson et al., 1989); and (*c*) a 1.5-kb insert from a partial rat lens cDNA clone encoding all of the putative gap junction protein connexin46 (Beyer et al., 1988; Paul, D. L., and D. A. Goodenough, manuscript in preparation). A fourth probe, containing the complete coding region of rat liver connexin26 (Zhang and Nicholson, 1989) was generated as follows: synthetic oligonucleotides corresponding to the amino- and carboxy-terminal sequences of rat liver connexin26 were used to amplify rat genomic DNA. After addition of Bgl II linkers, the amplified fragment was subcloned into the expression vector SP64T (Krieg and Melton, 1984), from which the connexin26-encoding insert was removed by digestion with Bgl II.

Immunofluorescent Localization of Connexin43

Cell cultures grown on uncoated glass coverslips were rinsed twice in serum-free medium before fixation for 1 h at room temperature in 1% formaldehyde (prepared freshly from paraformaldehyde) in PBS (final pH = 7.4). The fixed cultures were treated with PBS containing 0.2% Triton X-100 and 5% normal goat serum (PBS-T-NGS) for 30 min, then incubated overnight at 4°C with a 1:500 dilution of either anticonnexin43(252-271) serum or preimmune serum in the same buffer. After a 30-min wash in PBS-T-NGS, the cultures were incubated with 1:500 rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS-T for 2 h at room temperature. The coverslips were washed as before and then mounted onto glass slides with MOWIOL 4-88 mounting medium (Calbiochem-Behring Corp., La Jolla, CA). The cultures were photographed on a Zeiss Axioscope microscope fitted with the appropriate filters.

Results

Biosynthesis and Posttranslational Processing of Connexin43 in NRK Cells

Our previous studies demonstrated that connexin43 translated in a cell-free reticulocyte lysate system migrates on SDS-PAGE as a single, 42,000-M_r protein. Immunoprecipitation of connexin43 from chick lens epithelial cells metabolically labeled with [^{35}S]methionine, however, yields two species: a 42-kD protein that comigrates with the in vitro translation product and a more heterogeneous 44–46-kD species. We demonstrated that connexin43 is synthesized in lens cells as the 42,000-M_r protein but is converted to the upper molecular weight species by the post-translational addition of phosphate (Musil et al., 1990).

To determine whether connexin43 undergoes a similar maturation process in other cell types, we first examined the biosynthesis of connexin43 in NRK cells. These cells possess morphologically recognizable gap junctions (Willingham et al., 1979) and have been reported to be exceptionally well dye coupled (Atkinson et al., 1981). 3-d-old, newly confluent monolayers of NRK cells were metabolically labeled with [^{35}S]methionine for 4 h, lysed, and immunoprecipitated with antibodies affinity purified from rabbit anti-connexin43(252-271) serum. This antiserum, raised against a peptide whose sequence corresponds to amino acids 252–271 of rat connexin43, has been characterized previously (Musil et al., 1990; Beyer et al., 1989; Crow et al., 1990). Upon analysis by SDS-PAGE, two [^{35}S]methionine-labeled species were obtained that comigrated with the 42- and the

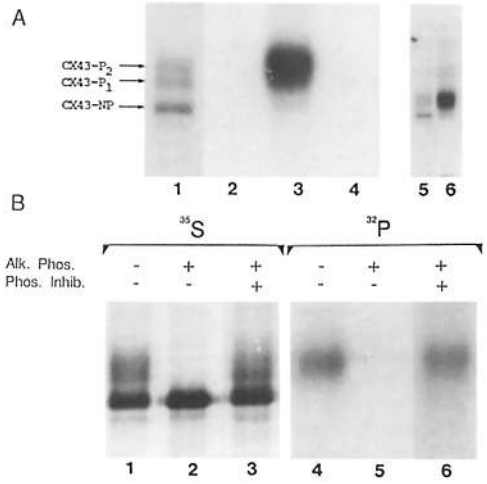

Figure 1. Phosphorylation of connexin43 in NRK cells. 3-d-old, newly confluent NRK cell cultures were metabolically labeled with either [^{35}S]methionine or [^{32}P]O_4 for 4 h before cell lysis and immunoprecipitation. (*A*) [^{35}S]methionine- (lanes *1* and *2*) or [^{32}P]O_4- (lanes *3* and *4*) labeled NRK cell lysates immunoprecipitated with affinity purified anticonnexin43(252-271) antibodies in either the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of 100 μg/ml of the synthetic connexin(252-271) peptide against which the antibodies were raised. The entire gel lanes from which lanes *1* and *3* were taken are shown in lanes *5* and *6*, respectively, and are not to scale with the rest of the figure. No [^{35}S]methionine- or [^{32}P]-labeled material other than connexin43 was recovered with the affinity-purified anticonnexin43(252-271) antibodies. (*B*) [^{35}S]-Methionine- or [^{32}P]O_4-labeled anticonnexin43(252-271) immunoprecipitates incubated for 4 h at 37°C in the presence of either alkaline phosphatase (lanes *2* and *5*), alkaline phosphatase plus an excess of phosphatase inhibitors (lanes *3* and *6*), or digestion buffer alone (lanes *1* and *4*). Exposure time of lanes *4* and *5* was 3 d. Lane *6* is from the same experiment but was analyzed 14 d later on a gel that was exposed to film for 6 d to compensate for the decay rate of the [^{32}P]isotope.

44–46-kD forms of connexin43 immunoprecipitated from chick lens epithelial cells (Fig. 1 *A*, lane *1*). Neither species was immunoprecipitated in the presence of an excess of the immunizing connexin43(252-271) peptide (lane *2*) or by preimmune serum (data not shown). The better resolution of the polyacrylamide gel system used in the current study revealed that the 44–46-kD species was actually a closely spaced but distinct doublet. Crow et al. (1990) have reported recovery of a similar triplet of proteins from vole fibroblasts immunoprecipitated with either the anticonnexin43(252-271) serum or with either of two additional antisera directed against other regions of rat connexin43. Before immunoprecipitation, we routinely boil the cell lysates in 0.6% SDS to denature proteins. Thus, the two higher relative molecular weight forms of connexin43 are likely to be directly recognized by the anticonnexin43(252-271) antibodies rather than indirectly coimmunoprecipitated by virtue of association with the 42-kD species. A similar conclusion can be drawn from Western blot analysis, which demonstrates reactivity of the anticonnexin43(252-271) serum with all three forms of connexin43 after separation on SDS-PAGE (data not shown; Crow et al., 1990).

The slower migrating forms of connexin43, but not the 42-kD species, could be metabolically labeled with [^{32}P]orthophosphate in intact cells (Fig. 1 *A*, lane *3*). Treatment of immunoprecipitated connexin43 with alkaline phosphatase completely removed the radiolabel from ^{32}P-connexin43 (Fig. 1 *B*, lane *5*), and quantitatively converted [^{35}S]methionine-labeled connexin43 from a triplet to a single 42-kD band (Fig. 1 *B*, lane *2*). Control experiments in which connexin43 samples were incubated in digestion buffer alone (Fig. 1 *B*, lanes *1* and *4*) or with alkaline phosphatase in the presence of phosphatase inhibitors (Fig. 1 *B*, lanes *3* and *6*) confirmed that this effect was due to dephosphorylation of connexin43 rather than to nonspecific proteolysis. Thus, the difference in apparent molecular mass between the 42-kD and the slower migrating forms of connexin43 was due solely to the presence of phosphate. Pulse-chase analysis revealed that connexin43 was initially synthesized in NRK cells as the 42-kD species and was converted first to the lower and then to the upper band of the phosphorylated doublet within 1–3 h (see Fig. 4). Together, these findings demonstrated that NRK cells process connexin43 in a manner similar to, although somewhat slower than, chick lens epithelial cells. Comparable results were obtained with fibroblastic baby hamster kidney cells (data not shown). Posttranslational phosphorylation of connexin43 is thus not confined to avian or lens cells but appears to be a general event in the biosynthesis of connexin43 in communication-competent cell types.

The relative electrophoretic mobility of connexins is notoriously dependent on the composition of the gel used in SDS-PAGE analysis (Green et al., 1988). To facilitate comparison of connexin43 species examined under different electrophoretic conditions, we will refer to the three connexin43 bands we observe by descriptive names rather than by their apparent molecular weights in our particular gel system. The primary translation product of connexin43 (the "42-kD form") will henceforth be referred to as connexin43-NP (not phosphorylated). The lower and upper bands of the phosphorylated connexin43 doublet will be designated connexin43-P_1 and connexin43-P_2, respectively. It is emphasized that the P_1/P_2 nomenclature does not indicate the number of phosphate residues attached to each form since the stoichiometry of phosphorylation is unknown.

Expression of Connexin43 in Cell Lines Deficient in Gap Junctional Communication

Although present in most animal tissues and cell lines derived from them, gap junctions are morphologically and physiologically lacking in certain cell types (Loewenstein, 1979; Azarnia et al., 1981; Pitts, 1978). We were interested in determining whether such communication-deficient cells synthesize connexins, and, if so, why they lack functional gap junctions. Two communication-deficient cell lines were examined: L929 cells, a subclone of mouse fibroblastic L cells previously used as a negative control in studies of metabolic gap junctional cell–cell coupling (Pitts, 1971; Larson and Sheridan, 1985), and mouse sarcoma S180 cells (Dunham and Stewart, 1953), known to be severely deficient in gap junction-mediated dye transfer and electrical coupling when grown under standard culture conditions (Furshpan and Potter, 1968; Mege et al., 1988).

We first examined whether these cell types expressed

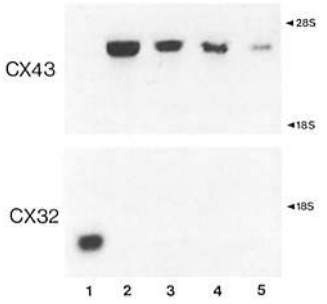

Figure 2. Northern blot analysis of connexin43 and connexin32 mRNA expression in communication-competent and -deficient cell lines. Total RNA was prepared from adult rat liver (lane *1*) or heart (lane *2*), and from 3-d-old, newly confluent monolayers of NRK (lane *3*), S180 (lane *4*), or L929 (lane *5*) cells. 10 μg of RNA were loaded in each lane and probed by hybridization at high stringency with [^{32}P]-labeled rat heart connexin43 cDNA (*top*) or rat liver connexin32 cDNA (*bottom*) as described in Materials and Methods. Arrowheads on the right indicate the positions of the 28S and 18S rRNA subunits.

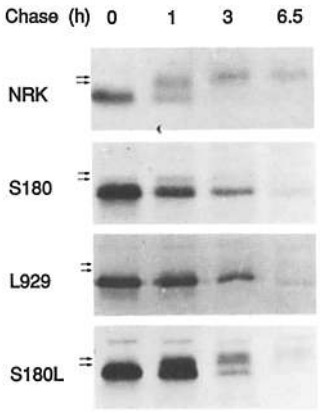

Figure 4. Pulse–chase analysis of the synthesis and post-translational processing of connexin43 in communication-competent and -deficient cell lines. 3-d-old, newly confluent cultures of NRK, S180, L929, or S180L cells were metabolically labeled with [^{35}S]methionine for 40 min and chased for 0, 1, 3, or 6.5 h in the presence of an excess of unlabeled methionine. The cultures were then lysed, immunoprecipitated with anticonnexin43(252-271) antibodies, and analyzed by SDS-PAGE and fluorography. The top and bottom arrows at the left of each panel denote the positions of connexin43-P_2 and connexin43-P_1, respectively. Note that S180 and L929 cells have no detectable [^{35}S]methionine-connexin43 migrating at the connexin43-P_2 position and that L929 cells lack connexin43-P_1 as well.

connexin43-encoding mRNA by Northern blotting. Total RNA was prepared from 3-d-old, newly confluent cultures of L929, S180, and NRK cells and 10-μg aliquots of each RNA were electrophoretically separated before transfer to a nylon membrane. The blot was then hybridized at high stringency with a ^{32}P-labeled cDNA probe containing the entire coding sequence of rat connexin43 (Beyer et al., 1987). As shown in Fig. 2, a single band of ~3.0 kb was obtained from all three cell lines (Fig. 2 *A*, lanes *3–5*). This species comigrated with authentic connexin43 mRNA from adult rat heart RNA (lane *2*) and was absent from RNA prepared from rat liver (lane *1*), a tissue known to lack detectable connexin43 (Beyer et al., 1987, 1989). Although precise quantitation was not attempted, densitometric scanning of the data shown in Fig. 2 indicated that the steady-state ratio of connexin43-related message in equal amounts of total RNA from rat heart, NRK, S180, and L929 cells was 1.0:0.56:0.47:0.21, respectively. In contrast, no specific signal was observed in the NRK, S180, and L929 samples when the blot was stripped and rehybridized under identical conditions with ^{32}P-labeled DNA probes encoding either rat liver connexin32 (B), rat liver connexin26, or rat lens connexin46 (data not shown). Rat liver connexin32 and connexin26 cDNAs have previously been shown to hybridize to mouse mRNA under high stringency conditions (Zhang and Nicholson, 1989), and the rat lens connexin46 probe cross-reacts with bovine mRNA transcripts under similar conditions (Paul, D. L., and D. A. Goodenough, manuscript in preparation). Lack of reactivity of our rat probes with mouse S180 and L929 cells is therefore unlikely to be due to species-specific differences in connexin sequences. We conclude that gap junctional communication-deficient S180 and L929 cells synthesize considerable amounts of mRNA encoding connexin43 or a very homologous protein but do not express detectable amounts of other well-characterized mammalian connexins.

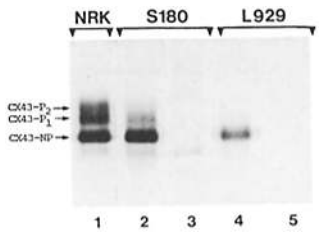

Figure 3. Synthesis of connexin43 in communication-deficient S180 and L929 cells. 3-d-old, newly confluent cultures of NRK (lane *1*), S180 (lanes *2* and *3*), or L929 (lanes *4* and *5*) cells were metabolically labeled with [^{35}S]methionine for 4 h before immunoprecipitation with affinity-purified anti-connexin43(252-271) antibodies in either the absence (lanes *1*, *2*, and *4*) or presence (lanes *3* and *5*) or 100 μg/ml of competing connexin43(252-271) peptide.

To determine whether the connexin43-related mRNA in S180 and L929 cells was actually translated, 3-d-old, newly confluent cultures of these cells were metabolically labeled with [^{35}S]methionine for 4 h before cell lysis and immunoprecipitation with affinity-purified anticonnexin43(252-271) antibodies (Fig. 3). A protein that comigrated with connexin43-NP from NRK cells (lane *1*) was specifically immunoprecipitated from both communication-deficient lines (lanes *2* and *4*). Significantly, however, these cells synthesized little (S180 cells) or no (L929 cells) detectable higher molecular weight connexin43 species. This can best be appreciated by comparing immunoprecipitates containing approximately equal (by laser densitometry) amounts of [^{35}S]methionine-labeled connexin43-NP (NRK and S180 cells, Fig. 3, lanes *1* vs. *2*; NRK and L929 cells, Fig. 5, lanes *1* vs. *6*). Comparison of the electrophoretic mobilities of the connexin43 bands recovered from S180 cells with those obtained from NRK cells revealed that S180 cells totally lacked the connexin43-P_2 form. The absolute amount of connexin43-P_1 produced in S180 cells was somewhat dependent on the stock of cells used but was always less than that synthesized by NRK cells.

The origin and fate of the anticonnexin43(252-271) immunoprecipitable protein recovered from S180 and L929 cells was examined in a pulse–chase experiment (Fig. 4). Confluent cultures of S180, L929, or, for comparison, NRK cells were metabolically labeled with [^{35}S]methionine for 40 min and then chased in the presence of an excess of unlabeled methionine for up to 6.5 h. The apparent molecular weight and amount of connexin43 present after the various chase periods was assessed by immunoprecipitation with anticonnexin43(252-271) antibodies followed by SDS-PAGE. Fig. 4 shows that in NRK cells, modification of newly synthesized connexin43-NP to the connexin43-P_1 form was well un-

derway after the first hour of chase and that subsequent conversion to the connexin43-P_2 species was largely complete within three hours. In contrast, in S180 cells only minor amounts of connexin43 were chased to the connexin43-P_1 form, with no detectable conversion to connexin43-P_2. L929 cells possessed neither immunoprecipitable connexin43-P_1 nor connexin43-P_2 at any time point. Densitometric analysis of several pulse–chase experiments conducted with three day-old, confluent monolayers of NRK cells revealed that one-half of the connexin43 synthesized during a 40-min pulse was degraded after 2–2.5 h of chase. Comparable rates of connexin degradation have been observed in other communication-competent cells such as primary cultures of chick lens epithelial cells ($t_{1/2}$ connexin43 = 1.5 h; Musil, L. S., unpublished) and embryonic mouse hepatocytes ($t_{1/2}$ connexin32 = 2.5–3.0 h [Traub et al., 1987] or 1.3–2.0 h [Traub et al., 1989]; $t_{1/2}$ connexin26 = 1.3–2.0 h [Traub et al., 1989]). The half-life of connexin43 in S180 and L929 cells was similar (1.5–2.0 h) (Fig. 4). These results rule out the possibility that S180 and L929 cells degrade connexin43 at a rate faster than conversion from the connexin43-NP to the connexin43-P_2 form can take place. The absence of connexin43-P_2 in these cells is therefore most likely due to a direct lack of processing of connexin43 to the P_2 form.

Phosphorylation of Connexin43 in Cell Lines Deficient in Gap Junctional Communication

In both chick lens epithelium (Musil et al., 1990) and rat NRK cells (Fig. 1), conversion of connexin43-NP to the connexin43-P_1 and P_2 species is due to the posttranslational addition of phosphate residues. The paucity of upper molecular weight forms of connexin43 in S180 and L929 cultures implied a deficiency in connexin43 phosphorylation in these cells. This was confirmed by metabolic labeling of S180, L929, and NRK cells with [^{32}P]orthophosphate for 4 h followed by immunoprecipitation with the anticonnexin43(252-271) antibodies (Fig. 5). When normalized to the amount of [^{35}S]methionine-labeled connexin43 immunoprecipitated from each cell line, S180 cells produced approximately one-sixth (lane *4*) as much immunoprecipitable ^{32}P-labeled material as did NRK cells (lane *2*) as determined by laser densitometry of linearly exposed fluorographs. No consistently detectable ^{32}P-labeled material was recovered from L929 cells (lane *7*). The overall protein phosphorylating capabilities of the three cell lines were similar as assessed by the amount of [^{32}P] incorporated into total cellular proteins per confluent culture (data not shown). Resolving between the P_1 and P_2 connexin43 species after [^{32}P]orthophosphate labeling was difficult due to the high energy of the [^{32}P] radioisotope. The majority of the ^{32}P-labeled connexin43 recovered from S180 cells appeared, however, to migrate somewhat faster than that from NRK cells, as expected if NRK cells phosphorylated connexin43-NP to both the P_1 and P_2 species whereas S180 cells produced only the connexin43-P_1 form. A faint ^{32}P-labeled band that migrated slightly slower than [^{35}S]methionine-connexin43-NP was specifically immunoprecipitated from both NRK and S180 cell lines. We do not know whether this material represents an additional, minor phosphorylated form of connexin43 or is the result of protein degradation or partial dephosphorylation during the immunoprecipitation procedure.

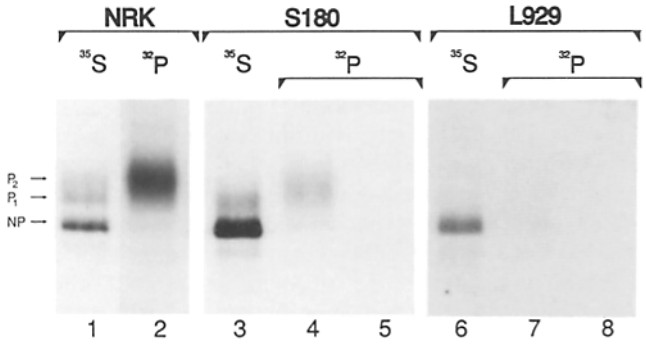

Figure 5. Metabolic labeling of connexin43 with [^{32}P]orthophosphate in S180 and L929 cells. S180, L929, and, for comparison, NRK cell cultures were incubated with either [^{35}S]-methionine (lanes *1*, *3*, and *6*) or [^{32}P]O_4 (lanes *2*, *4*, *5*, *7*, and *8*) for 4 h, lysed, and immunoprecipitated with affinity-purified anticonnexin43(252-271) antibodies. Nonspecific binding of the antibodies was assessed by immunoprecipitation in the presence of 100 μg/ml of connexin43(252-271) competing peptide (lanes *5* and *8*). Lanes *6–8* were exposed twice as long as the other lanes to increase the intensity of the connexin43 signal.

Increasing intracellular levels of cAMP (Azarnia et al., 1981; Loewenstein, 1985) or catalytically active cAMP-dependent protein kinase (Wiener and Loewenstein, 1983) stimulates gap junction–mediated communication between fibroblastic cells. Conversely, activation of the protein tyrosine kinase pp60$^{v\text{-}src}$ sharply reduces junctional permeability (Atkinson et al., 1981; Azarnia et al., 1988). Our finding that connexin43 itself is a phosphoprotein raised the possibility that connexin43 was phosphorylated on serine or threonine residues (by cAMP-dependent protein kinases) in communication-competent cells, but on tyrosine residues in at least some communication-deficient cell lines such as S180 cells. This was examined by metabolically labeling S180 and NRK cells for 4 h with [^{32}P]orthophosphate and determining the nature of the radiolabeled residue(s) in connexin43 by immunoprecipitation followed by one-dimensional phosphoamino acid analysis. In both cell lines, the ^{32}P-labeled material comigrated with the phosphoserine internal standard with no trace of either phosphothreonine or phosphotyrosine (Fig. 6). Thus, phosphorylation of connexin43 in NRK and in S180 cells differed in the quantity, but not the type, of amino acid modified. The defect in gap junction–mediated intercellular communication in S180 cells must therefore be due to something other than the direct action of a tyrosine protein kinase on connexin43.

Phosphorylation of Connexin43 in Communication-competent S180 Transfectants

Our data demonstrate that NRK and lens epithelial cells phosphorylate connexin43 to the P_2 form whereas communication-deficient S180 and L929 cells do not. Differential phosphorylation of connexin43 could either be of functional significance or be a trivial result due to inherent differences between cell types (such as species differences) unrelated to their capacity for junctional communication. To help distinguish between these two possibilities, we examined the biosynthesis of connexin43 in a line of S180 cells (S180L) that had been converted to a communication-competent phenotype by transfection with a chicken cDNA encoding the cell–

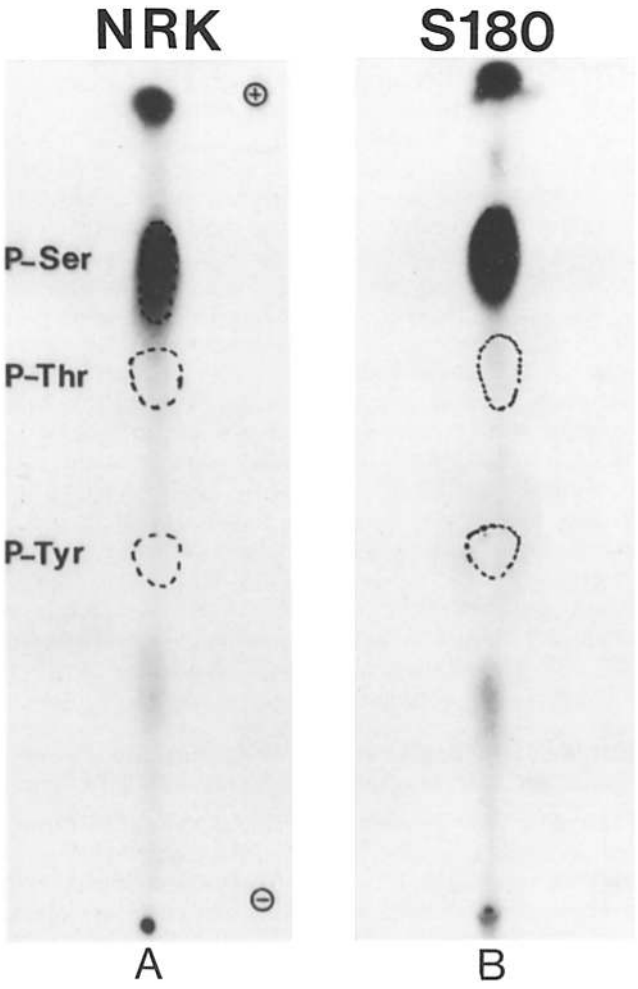

Figure 6. Phosphoamino acid analysis of metabolically labeled [^{32}P]-connexin43 from NRK and S180 cells. 3-d-old, newly confluent cultures of NRK (*left*) or S180 (*right*) cells were incubated for 4 h with [^{32}P]O_4 and then lysed. Connexin43 was immunoprecipitated from the cell lysates with anticonnexin43(252-271) antibodies, purified by SDS-PAGE, and hydrolyzed before one-dimensional phosphoamino acid analysis as described in Materials and Methods. Electrophoresis was from the negative to the positive electrode. The positions of the phosphoamino acid internal standards are outlined.

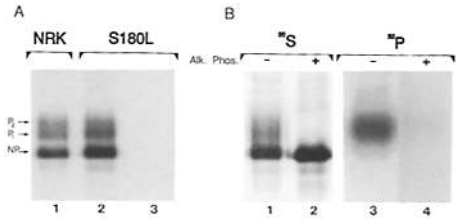

Figure 7. Phosphorylation of connexin43 in communication-competent S180L cells. 3-d-old, newly confluent monolayers of S180L cells were labeled with [^{35}S]methionine (*A*, lanes *2* and *3*; *B*, lanes *1* and *2*) or [^{32}P]O_4 (*B*, lanes *3* and *4*) for 4 h. The cells were lysed and immunoprecipitated with affinity purified anticonnexin43(252-271) antibodies. (*A*) Immunoprecipitation in either the absence (lane *2*) or presence (lane *3*) of 100 μg/ml of competing connexin-(252-271) peptide. [^{35}S]Methionine-connexin43 immunoprecipitated from a parallel culture of NRK cells is shown in lane *1* for comparison. (*B*) The immunoprecipitates were incubated for 4 h at 37°C in either the absence (*B*, lanes *1* and *3*) or presence (*B*, lanes *2* and *4*) of alkaline phosphatase before electrophoresis.

cell adhesion molecule L-CAM (Mege et al., 1988) (Fig. 7). Expression of L-CAM allows these cells to form stable intercellular contacts, resulting in the formation of epithelial-like sheets of flattened polygonal cells morphologically very different from the round and spindle-shaped parental cells. Concomitant with this change in cell shape is a dramatic increase in the extent of morphologically recognizable gap junctions and in the ability to transfer Lucifer yellow between adjoining cells (Mege et al., 1988). Northern blot analysis indicated that the steady-state connexin43 mRNA content of S180L cells is similar to that of the parental S180 line (data not shown). Metabolic labeling of 3-d-old, newly confluent cultures of S180L cells with [^{35}S]methionine for 4 h followed by immunoprecipitation with affinity-purified anticonnexin43(252-271) antibodies revealed NP, P_1, and P_2 forms of connexin43 (Fig. 7 *A*, lane *2*) in amounts comparable to those recovered from similarly labeled NRK cells (Fig. 7 *A*, lane *1*). The slower migrating forms of connexin43, but not the connexin-43-NP band, could be metabolically labeled with [^{32}P]orthophosphate (Fig. 7 *B*, lane *3*) and were sensitive to alkaline phosphatase digestion (Fig. 7 *B*, lanes *2* and *4*). Pulse–chase analysis demonstrated that conversion of connexin43-NP to the slower migrating forms occurred at a rate comparable to that observed in NRK cells, as did connexin43 degradation (Fig. 4). The P_2 form of connexin43 immunoprecipitated from S180L cells was thus indistinguishable from that recovered from NRK cells. Together, these results are consistent with a relationship between phosphorylation of connexin43 to the P_2 form and the ability of connexin43-expressing cells to communicate via gap junctions.

Effect of Disruption of Junctional Communication on Phosphorylation of Connexin43

Incubation of confluent S180L monolayers for 2 h with antichicken L-CAM Fab′ fragments causes the cells to completely dissociate from each other (but not from the substratum) and to revert to the parental S180 cell morphology (Mege et al., 1988). Not surprisingly, this disruption in cell–cell contact is accompanied by a sharp decrease in junctional dye transfer. If gap junctional communication were indeed correlated with connexin43-P_2 formation, then we would expect that S180L cells dissociated by anti-L-CAM Fab′ fragments would no longer phosphorylate connexin43 to this form. This prediction was borne out by an experiment in which S180L cells were metabolically labeled with [^{35}S]methionine for 40 min and then chased for 4 h in the continuous presence of anti-L-CAM Fab′ fragments (Fig. 8). In control cultures incubated without antibody fragments, more than one-third of the immunoprecipitated [^{35}S]methionine-connexin43 migrated at the P_2 position (lane *1*). In contrast, no connexin43-P_2 was detectable in anti-L-CAM Fab′-treated cultures, even though the total amount of [^{35}S]methionine-labeled connexin43 recovered was identical to that from control cells (lane *2*). An equal concentration of irrelevant (goat anti-rabbit IgG) Fab′ fragments was un-

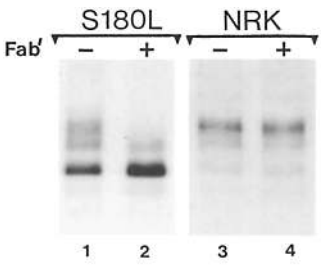

Figure 8. Anti-L-CAM Fab' fragments inhibit connexin43-P_2 formation in S180L but not NRK cells. Confluent cultures of S180L (lanes *1* and *2*) or NRK (lanes *3* and *4*) cells were metabolically labeled with [^{35}S]methionine for 40 min, after which they were chased for 4 h in either the absence (lanes *1* and *3*) or presence (lanes *2* and *4*) of 300 μg/ml rabbit anti-L-CAM Fab' fragments (Mege et al., 1988). Lysates prepared from these cultures were then immunoprecipitated with affinity purified anticonnexin43 (252-271) antibodies and analyzed by SDS-PAGE. Anti-L-CAM Fab' fragments markedly disrupted cell-cell association in S180L cultures within 40 min but had no effect on NRK cell adhesion.

able to disrupt S180L monolayers or to inhibit connexin43-P_2 formation (data not shown). The anti-L-CAM fragments did not alter either intercellular adhesion or connexin43 phosphorylation in NRK cells, ruling out the possibility that their effect on S180L cells was due to a nonspecific effect on cell adhesion and/or viability (lanes *3* and *4*). These findings suggest that the lack of labeled connexin43-P_2 in S180L cells pulsed and chased in the presence of anti-L-CAM Fab' fragments is a result of disruption of L-CAM-mediated cell–cell adhesion. Efforts to address whether anti-L-CAM Fab' treatment could cause dephosphorylation of previously synthesized connexin43-P_2 were inconclusive since dephosphorylation could not be unambiguously distinguished from inhibition of new phosphorylation.

We were next interested in determining whether other inhibitors of junctional communication also interfered with connexin43 phosphorylation. It has long been known that heptanol and octanol rapidly and reversibly block gap junctional communication in many cell types, including several shown to express connexin43 (e.g., cardiac myocytes [Burt and Spray, 1988]; chick lens epithelial cells [Miller and Goodenough, 1985]). Although the mechanism whereby these alcohols affect junctional permeability is obscure, they do not appear to act by perturbing cytosolic free Ca^{2+}, pH, or cAMP, nonjunctional membrane conductance, cell viability, or gap junction morphology (Chanson et al., 1989; Meda et al., 1988). Confluent monolayers of NRK cells were metabolically labeled for 40 min with [^{35}S]methionine and then chased for four hours in the presence of 3.5 mM heptanol, a concentration that blocks dye transfer between NRK cells (data not shown) and other cell types within 10 minutes (Burt and Spray, 1988; Miller and Goodenough, 1985; Chanson et al., 1989). As shown in Fig. 9, virtually no connexin43-P_2 was immunoprecipitated from heptanol-treated cells (lane *3*), whereas control cultures contained large amounts of this species (lane *2*). Heptanol also abolished metabolic labeling of connexin43-P_2 (but not P_1) with [^{32}P]orthophosphate (lane *5*) during a 4-h incubation, consistent with an effect of heptanol on phosphorylation of newly synthesized connexin43. Shorter [^{32}P]labeling periods were not examined since the turnover rate of phosphate on connexin43 is unknown, making interpretation of the results of such experiments difficult. Prolonged exposure to 3.5 mM heptanol did not appear to be toxic to NRK cells since incorporation of [^{35}S]methionine and [^{32}P]orthophosphate into total cellular proteins was unaffected for at least 5 h (data not shown), and because cultures incubated with heptanol for 4 h and then washed into heptanol-free medium were subsequently able to produce normal amounts of connexin43-P_2 (lane *1*). Unlike that induced by anti-L-CAM Fab' fragments, however, inhibition of junctional communication by heptanol appeared to be accompanied by a decrease in the metabolic stability of connexin43 (compare the total amount of immunoprecipitable connexin43 in Fig. 9, lane *2* with lane *3*). Qualitatively similar results were obtained with S180L cells (data not shown). In principle, the lack of detectable connexin43-P_2 in the presence of heptanol could be due to (*a*) a direct block in phosphorylation of connexin43 to the P_2 form; (*b*) an increase in the degradation rate of connexin43; and/or (*c*) heptanol-induced rapid dephosphorylation of connexin43-P_2. This last possibility is, however, unlikely because a 10–30-min chase in the presence of heptanol does not detectably reduce the level of immunoprecipitable connexin43-P_2 recovered from [^{35}S]methionine-labeled NRK cells (data not shown). Whatever the mechanism of heptanol action, these results are consistent with our previous observations that only cells with permeable gap junctions accumulate detectable levels of connexin43-P_2.

Morphological Localization of Connexin43 in Communication-competent and -deficient Cell Lines

The intracellular distribution of connexin43 in the various cell lines was examined by immunofluorescence using the anti-connexin43(252-271) serum. Formaldehyde fixed and detergent permeabilized NRK cells showed a pattern of discrete punctate immunoreactivity at cell–cell interfaces that was absent from unassociated cell surfaces (Fig. 10 *A*). Such areas of intense intercellular staining with the anticonnexin43(252-271) serum have been demonstrated by EM to represent bona fide gap junctions in several tissues (e.g., lens [Musil et al., 1990], heart [Beyer et al., 1989], and brain [Dermietzel et al., 1989]). The antiserum also reacted strongly with a juxtanuclear region of NRK cells. This Golgi apparatus–like staining, like that at the cell surface, was specific in that it was abolished when the anticonnexin43 (252-271) serum was substituted with preimmune serum

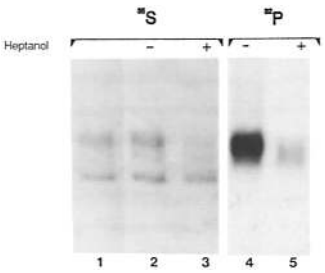

Figure 9. Effect of heptanol on phosphorylation of newly synthesized connexin43 in NRK cells. Lanes *1–3*: NRK cultures were metabolically labeled with [^{35}S]-methionine for 40 min and then chased for 4 h either with (lane *3*) or without (lanes *1* and *2*) heptanol. To test for irreversible toxic effects of heptanol, one culture was incubated with heptanol for 4 h and subsequently in heptanol-free medium for an additional hour before [^{35}S]methionine labeling (lane *1*). Lanes *4* and *5*: NRK cultures were metabolically labeled for 4 h with [^{32}P]orthophosphate in either the absence (lane *4*) or presence (lane *5*) of heptanol. All cultures were lysed and immunoprecipitated with affinity-purified anticonnexin43(252-271) antibodies. Incorporation of [^{32}P] into TCA precipitable total cellular proteins in the presence of heptanol was 80% of that in its absence.

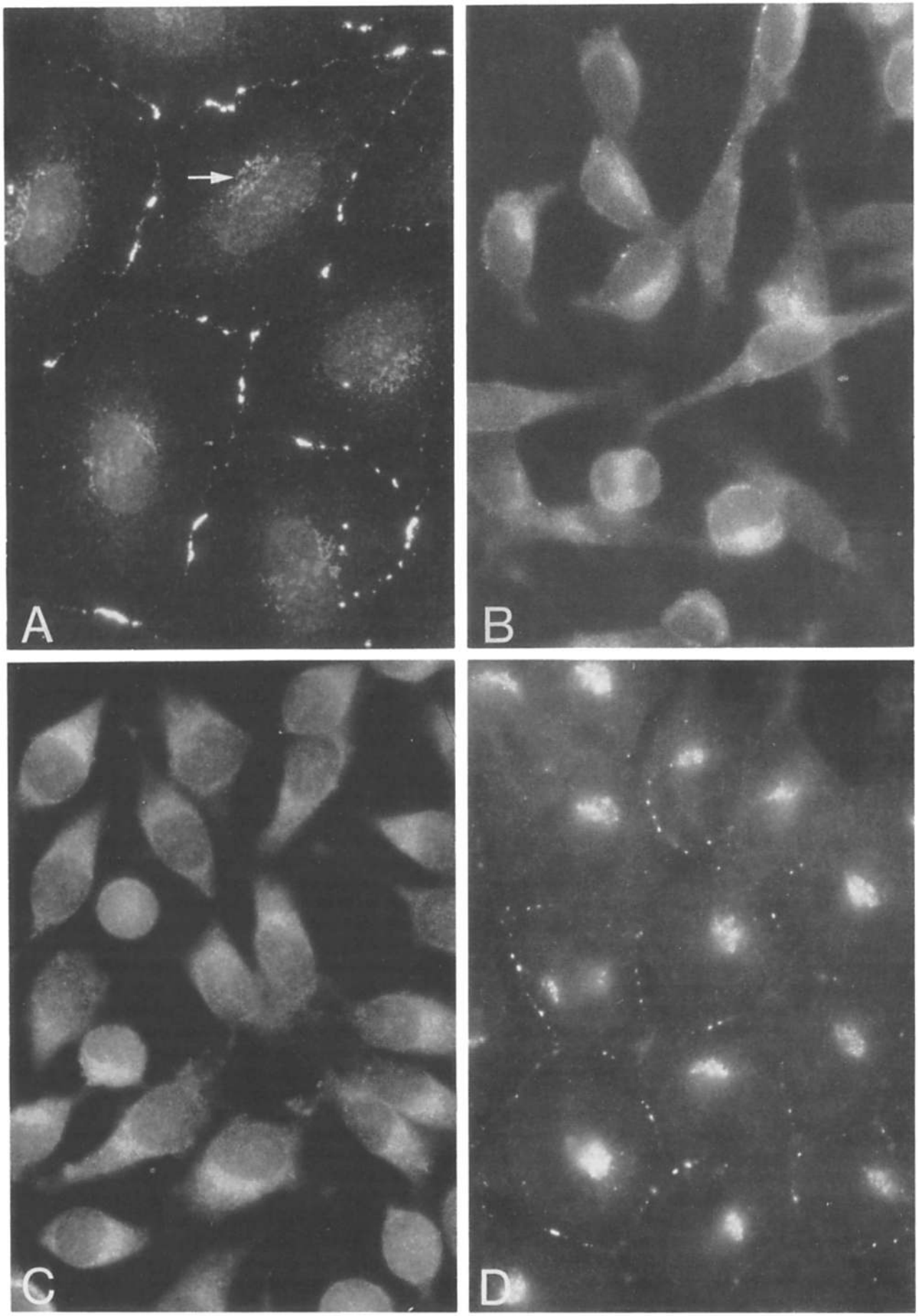

Figure 10. Immunohistochemical localization of connexin43 in communication-competent and -deficient cell lines. Formaldehyde fixed, detergent permeabilized cultures of NRK (*A*), S180 (*B*), L929 (*C*), or S180L (*D*) cells were stained with anticonnexin43(252-271) serum followed by rhodamine-labeled goat anti-rabbit IgG. Connexin43-specific staining was found intracellularly in all cell types but was localized to cell surface maculae only in communication-competent cells.

or with anticonnexin43(252-271) serum preabsorbed with 100 μg/ml of the immunizing connexin43(252-271) peptide (not shown). In contrast to NRK cells, communication-deficient S180 (Fig. 10 *B*) and L929 (Fig. 10 *C*) cells showed very few (S180) or no (L929) detectable immunoreactive sites between cells despite considerable specific intracellular staining. Failure to localize connexin43 at the plasma membrane of S180 cells was in keeping with their previously demonstrated lack of morphologically recognizable gap junctions at regions of cell–cell contact (Mege et al., 1988). The distribution of anticonnexin43(252-271) immunoreactivity in S180L cells resembled the pattern observed in NRK cells, with prominent punctate staining at cell–cell contact sites (Fig. 10 *D*). These results suggest that the S180 and L929 cell lines, unlike communication-competent cells, do not detectably accumulate connexin43 at the plasma membrane in gap junctions. The fact that these same cells also do not express connexin43-P_2 raises the possibility that phosphorylation of connexin43 to the P_2 form is involved in some aspect of gap junctional plaque formation (see Discussion).

Discussion

Although gap junctions were first described morphologically over two decades ago (reviewed in Bennett and Goodenough, 1978), it has only been in the last few years that the family of proteins that make up these cell-to-cell channels, the connexins, has been identified (Beyer et al., 1988). How connexins assemble to form gap junctions and the mechanisms that regulate their function are largely unknown. We have previously shown that connexin43 is efficiently posttranslationally phosphorylated in chick lens epithelial cells (Musil et al., 1990), a cell type possessing morphologically and physiologically recognizable gap junctions (Goodenough et al., 1980; Schuetze and Goodenough, 1982; Musil et al., 1990). In the current study, we have examined the post-translational processing of connexin43 in several other cell types that vary greatly in their ability to mediate gap junctional communication. Using a combination of biochemical and morphological techniques, we found that all of the cell types examined synthesized connexin43, including, surprisingly, those known to be severely deficient in junctional communication. Communication-competent and -deficient cell lines differed markedly, however, in their capacity to phosphorylate connexin43 and to accumulate it at the plasma membrane. Together, our results provide strong evidence that connexin phosphorylation is positively correlated with gap junctional communication.

Each of the junctional transfer-competent (NRK, S180L) and -deficient (S180, L929) cell lines examined synthesized immunoprecipitable connexin43 and degraded it at approximately equal rates. Processing of connexin43 in these two classes of cells was, however, very different in at least two respects. First, connexin43 was visualized in communication-competent cells at the cell surface in gap junctional maculae. In contrast, connexin43 was virtually undetectable at the surface of S180 and L929 cells; whether this was due to a block in transport to the plasma membrane or to a very rapid internalization rate is not currently known. Second, S180 and L929 cells, unlike communicating cells, did not detectably phosphorylate connexin43 to the connexin43-P_2 form despite their ability to phosphorylate other cellular proteins to normal levels. Transfection of S180 cells with a cDNA encoding the cell–cell adhesion molecule L-CAM corrected both the connexin43 phosphorylation and junctional communication defects. Lack of junctional transfer in parental S180 cells thus cannot be explained by a mutation in connexin43 that renders it inherently incapable of forming functional gap junctions. Ordinarily communication-competent cells could be converted to a communication-defective phenotype by exposure to either anti-L-CAM Fab' fragments or heptanol; as in S180 and L929 cells, no detectable connexin43-P_2 was produced under these conditions. Regardless of whether the lack of connexin43-P_2 in communication-deficient cells was due to a direct block in connexin43 phosphorylation or to an increased rate of connexin43 dephosphorylation or degradation, our results all support the same conclusion: gap junctional communication-competent cells accumulate detectable amounts of connexin43-P_2, whereas communication-deficient cells do not. The presence of connexin43-P_1 in S180 cells and its absence in L929 cells parallels the low but detectable junctional permeability of S180 relative to L929 cells (Mege et al., 1988). Although this might imply a limited role for the P_1 form of connexin43 in junctional communication, our data are more consistent with the possibility that S180 cells contain very low levels of connexin43-P_2 that cannot be detected by our current methodology.

Crow et al. (1990) have examined the biosynthesis of connexin43 in communication-competent rodent fibroblasts by pulse–chase analysis. Similar to the results presented here, they find that connexin43 in vole fibroblasts is synthesized as a single, M_r = ~43,000 protein that is posttranslationally converted to multiple, slower migrating species by the addition of phosphate onto serine residues. However, they also report a rapid (within 30–40 min of chase) reduction in the apparent molecular weight of the processed forms of connexin43 back to the M_r = 43,000 initial translation product, a shift they suggest (but do not directly demonstrate) to be due to irreversible dephosphorylation of connexin43. We have never observed such a post-translational increase in the electrophoretic mobility of connexin43 in any of the cell types we have examined (Fig. 4 and Musil et al., 1990). Since the anticonnexin43 serum used in our studies and those of Crow et al. (1990) is identical, we can only speculate that differences in the cell types or methodology used are responsible for the discrepancy in the findings between our two groups.

Phosphorylation has previously been implicated in the activity of connexin32. Saez et al. (1986) have reported that addition of 8-bromo-cAMP to paired rat hepatocytes increased gap junctional conductance by 50–75% and resulted in a 1.6-fold increase of incorporation of [^{32}P]orthophosphate into immunoprecipitable connexin32 within one hour. It was unclear, however, what fraction of the total connexin32 population ever became phosphorylated in intact cells and whether the enhanced labeling of connexin32 with [^{32}P] in the presence of cAMP reflected an actual increase in new phosphorylation or merely a stimulation of the turnover rate of phosphate on already phosphorylated molecules. The issue of whether a decrease in cell–cell coupling was associated with a drop in connexin32 phosphorylation was not addressed, making it difficult to assess the functional rela-

tionship, if any, between connexin32 phosphorylation and junctional communication. In contrast to connexin32, the extent of phosphorylation of connexin43 is readily visualized due to the shift in apparent molecular weight that takes place upon phosphate addition. Our results establish an unambiguous correlation between phosphorylation of connexin43 and gap junctional communication, whereas published evidence does not permit a similar conclusion to be drawn for connexin32.

Our demonstration of connexin43 in cells lacking morphologically and physiologically recognizable gap junctions illustrates that connexin synthesis cannot be taken as proof that a cell possesses functioning intercellular channels. Furthermore, the presence of detectable connexin43-P_2 does not guarantee that cells are actively communicating. For example, heptanol-treated NRK cells are largely uncoupled from one another but retain previously synthesized connexin43-P_2; apparently heptanol, which inhibits the accumulation of newly synthesized connexin43-P_2 (Fig. 9), does not induce detectable dephosphorylation of previously synthesized connexin43-P_2. Thus biochemical analysis of connexin content cannot currently substitute for physiological assessment of junctional conductance as an assay for gap junction–mediated cell–cell coupling.

What effect does cell–cell adhesion have on connexin43 biosynthesis and processing? When grown under standard culture conditions, the junctional communication-deficient cell lines examined are severely lacking in intercellular adhesion structures (Mege et al., 1988; Matsuzaki et al., 1990; Nagafuchi et al., 1987) whereas cells of the communicating lines are tightly bound to each other. The fact that S180 and L929 cells synthesize high levels of connexin43 demonstrates that tight cell–cell association is not a requirement for connexin expression, in keeping with studies demonstrating translation of exogenous connexin-encoding mRNAs in single, unpaired *Xenopus* oocytes (Swenson et al., 1989). Our results also suggest that connexin43 is as metabolically stable in the absence as in the presence of CAM-mediated cell–cell adhesion (i.e., compare the degradation half-time of connexin43 in S180 and in S180L cells); this is in contrast to several other proteins involved in intercellular interactions (reviewed in Stevenson and Paul, 1989). Expression of L-CAM caused S180L cells to phosphorylate connexin43 to the P_2 form as well as to bind tightly to each other. Strong cell–cell association may thus be important for connexin43-P_2 production, as it appears to be for gap junction formation (the "precedence hypothesis"; Edelman, 1988) (Keane et al., 1988).

The role of connexin43 phosphorylation in gap junction formation and function is unknown. Modification of connexin43 to the P_2 form might be involved in connexin oligomerization, transport to or from the plasma membrane, assembly of gap junction plaques, or gating of the junctional channel. We are currently investigating the intracellular transport and assembly of connexin43 and how these events relate to modification of connexin43 to the P_2 form. It is likely that identification and site-directed mutagenesis of the serine residue(s) involved in connexin43-P_2 formation will be required before the significance of connexin43 phosphorylation can be definitively established.

We thank Dr. David Paul for providing the connexin DNA clones, Dr. Kathryn Crossin for the purified anti-L-CAM Fab' fragments, and Dr. Katherine Swenson for help with the phosphoamino acid analysis.

This work was supported by National Institutes of Health (NIH) grants GM-18974 and EY-02430 (to D. A. Goodenough) and by NIH grants HD09635 (to G. Edelman) and HD-16550 (to B. Cunningham).

Received for publication 29 May 1990 and in revised form 13 August 1990.

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