Biochemical Analysis of Connexin43 Intracellular Transport, Phosphorylation, and Assembly into Gap Junctional Plaques

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Abstract. We previously demonstrated that the gap junction protein connexin43 is translated as a 42-kD protein (connexin43-NP) that is efficiently phosphorylated to a 46,000-M_r species (connexin43-P_2) in gap junctional communication-competent, but not in communication-deficient, cells. In this study, we used a combination of metabolic radiolabeling and immunoprecipitation to investigate the assembly of connexin43 into gap junctions and the relationship of this event to phosphorylation of connexin43. Examination of the detergent solubility of connexin43 in communication-competent NRK cells revealed that processing of connexin43 to the P_2 form was accompanied by acquisition of resistance to solubilization in 1% Triton X-100. Immunohistochemical localization of connexin43 in Triton-extracted NRK cells demonstrated that connexin43-P_2 (Triton-insoluble) was concentrated in gap junctional plaques, whereas connexin43-NP (Triton-soluble) was predominantly intracellular. Using either a 20°C intracellular transport block or cell-surface protein biotinylation, we determined that connexin43 was transported to the plasma membrane in the Triton-soluble connexin43-NP form. Cell-surface biotinylated connexin43-NP was processed to Triton-insoluble connexin43-P_2 at 37°C. Connexin43-NP was also transported to the plasma membrane in communication defective, gap junction–deficient S180 and L929 cells but was not processed to Triton-insoluble connexin43-P_2. Taken together, these results demonstrate that gap junction assembly is regulated after arrival of connexin43 at the plasma membrane and is temporally associated with acquisition of insolubility in Triton X-100 and phosphorylation to the connexin43-P_2 form.

GAP junctions are plasma membrane specializations that mediate the regulatable transfer of small molecules and ions between adjoining cells (Gilula et al., 1972; Loewenstein, 1981; Beyer et al., 1990). Present in virtually all metazoan tissues, gap junctions are involved both in relaying signals and in maintaining metabolic continuity between connected cells. In electrically excitable tissues including myocardium, smooth muscle, and nerve, gap junctions provide low-resistance electrical pathways between cells that are essential for their specialized functions (De Mello, 1987). Gap junction–mediated intercellular communication has also been implicated in fundamental cellular processes such as embryonic development, differentiation, and growth control (Loewenstein, 1979; Mehta et al., 1986; Guthrie and Gilula, 1989).

It has been well established that gap junctions in vertebrates are comprised of connexins, members of a family of closely related integral membrane proteins (Stevenson and Paul, 1989). The events involved in the assembly of newly synthesized connexin monomers into functional gap junctional plaques are, however, poorly understood. A necessary step in junction formation is oligomerization of six connexin monomers into half an intercellular channel (a connexon) in an as yet undefined cellular compartment. A connexon in the plasma membrane of one cell must then join with a connexon in an opposing cell membrane to form an intercellular channel. These channels become concentrated at cell–cell interfaces into very high-density clusters ($\sim 10^4$ channels/μm^2 of membrane) referred to as gap junctional plaques or maculae (Loewenstein, 1981; Yamasaki, 1990). It is not known how these various assembly steps are regulated, or whether they serve as control points in the establishment of gap junction–mediated cell–cell communication.

Many effectors of protein kinases modulate gap junctional communication, and it has recently been demonstrated that certain connexins themselves are phosphorylated (reviewed by Musil and Goodenough, 1990; Stagg and Fletcher, 1990). Saez et al. (1986) reported that addition of 8-bromo-cAMP to primary rodent hepatocytes results in a 1.6-fold increase in incorporation of ^{32}P into immunoprecipitable connexin32 within 30–60 min, apparently without an appreciable increase in the total amount of connexin32 protein (Traub et al., 1987). Since gap junctional conductance was also increased 50–75% during this period, it was proposed that phosphorylation of connexin32 may be involved in the regulation of gap junctional communication. Within the last year several groups have demonstrated serine phosphorylation of connexin43, a connexin first identified in heart yet present also in a wide variety of other tissues (Crow et al., 1990; Musil et al., 1990*a*, *b*; Filson et al., 1990; Swenson et al.,

1990; Laird et al., 1991). Agonists of protein kinase A and protein kinase C have been shown to affect gap junctional conductance in several connexin43-containing cell types (Loewenstein, 1985; Stagg and Fletcher, 1990; Spray and Burt, 1990); as with connexin32, the mechanism whereby these agents influence cell–cell communication is not known.

A potential functional relationship between serine phosphorylation of connexin43 and gap junctional communication was suggested by studies examining the posttranslational processing of connexin43 in cell lines that differ greatly in their ability to form morphologically and physiologically recognizable gap junctions (Musil et al., 1990*b*). In all gap junctional communication-competent cell types examined, connexin43 is synthesized as a single, 42-kD species that is converted to a species of ~44-kD (connexin43-P_1) and then to one of ~46 kD (connexin43-P_2) by the addition of phosphate onto serine residues. In contrast, certain cell lines that are severely deficient in junctional communication (mouse S180 and L929 cells) constitutively synthesize connexin43 but neither process it to the P_2 form nor accumulate connexin43 in visible gap junctional plaques. Conversion of S180 cells to a communication-competent phenotype by transfection with a cDNA encoding the cell–cell adhesion molecule L-CAM induces both phosphorylation of connexin43 to the P_2 form and assembly of gap junctional plaques. In complementary experiments, ordinarily communication-competent cells treated with known inhibitors of gap junction permeability no longer detectably process connexin43 to connexin43-P_2. These results establish a strong correlation between the ability of cells to phosphorylate connexin43 to the P_2 form and to form morphologically and physiologically recognizable gap junctions, but shed little light on the functional role of connexin43 phosphorylation.

In the current study we investigated further the relationship between connexin43 phosphorylation, gap junction assembly, and cell–cell communication. Biochemical assays for transport of connexin43 to the plasma membrane and for accumulation of connexin43 in junctional plaques were developed and used to analyze connexin43 processing in various cell types. In NRK and other communication-competent cells, connexin43 undergoes a dramatic posttranslational change in Triton X-100 solubility that is temporally associated both with phosphorylation of connexin43 to the P_2 form and with assembly of connexin43 into gap junctional plaques. Both acquisition of Triton insolubility and phosphorylation of connexin43 occur (at least in part) after transport of connexin43 to the plasma membrane. Communication-defective, gap junction–deficient S180 and L929 cells also transport connexin43 to the cell surface but do not process it to Triton-insoluble, terminally phosphorylated connexin43-P_2. Processing of connexin43 to the P_2 form is thus not required for transport of connexin43 to the cell surface but is tightly correlated with incorporation of connexin43 into junctional plaques, a fact suggesting that terminal phosphorylation of connexin43 may be involved in a later step in gap junctional plaque assembly or in functional processes.

Materials and Methods

Reagents

Tissue culture reagents were purchased from Gibco (Grand Island, NY), except for FCS, which was purchased from HyClone (Logan, Utah). [^{35}S]Methionine (cell-labeling grade) was from New England Nuclear (Boston, MA); Tran^{35}S-label was obtained from ICN Radiochemicals (Div. ICN Biomedicals Inc., Irvine, CA). NHS–LC-biotin and avidin-agarose beads were purchased from Pierce Chemical Co. (Rockford, IL). Lauryldimethylamine oxide (LDAO) was from Calbiochem Corp. (San Diego, CA). Unless otherwise specified, all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

The NRK, S180, and L929 cell lines were maintained as previously described (Musil et al., 1990*b*). Three-day-old, newly confluent 60-mm cultures were used for all experiments unless otherwise specified. The gap-junctional communication-competence of the NRK cells 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).

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

Details of the metabolic labeling of cell cultures with [^{35}S]methionine are given elsewhere (Musil et al., 1990*a*). Each 60-mm cell culture was labeled with 100 μCi/ml of [^{35}S]methionine, except in the case of cell-surface biotinylation experiments for which 350 μCi/ml of [^{35}S]methionine or Tran^{35}S-label were used. At the end of the labeling or chase period, the cultures were solubilized in the presence of 0.6% SDS, and the resulting cell lysates were immunoprecipitated according to the procedure of Musil et al. (1990*a*). The anti-connexin43 antibodies used throughout this study were affinity purified from a rabbit antiserum generated against a connexin43-specific synthetic peptide encoding amino acids 252–271 of rat heart connexin43 (Beyer et al., 1989) as previously described (Musil et al., 1990*b*).

SDS Gel Electrophoresis and Fluorography

Immunoprecipitated samples were analyzed on 10% SDS-polyacrylamide gels (Laemmli, 1970). Gels were processed for fluorography with EN^{3}HANCE (New England Nuclear, Boston, MA) using the supplier's suggested protocol and then exposed to prefogged XAR-5 film (Eastman Kodak Co., Rochester, NY). Quantitative densitometry was conducted with an UltroScan XL laser densitometer (LKB Instruments Inc., Broma, Sweden).

Detergent Solubilization of Connexin43 from Cultures and Lenses

Metabolically labeled cell cultures were scraped from the tissue culture dish with a rubber spatula into 4 ml of Leibovitz's L-15 medium supplemented with 2 mM PMSF and 10 mM *N*-ethylmaleimide (4°C) and then pelleted by centrifugation at 150 *g* for 7 min. Lenses were dissected from 10-d white leghorn chicken embryos (taking care to remove associated ciliary epithelial cells) and metabolically labeled with [^{35}S]methionine as described elsewhere (Musil et al., 1990*a*).

In the standard Triton X-100 solubilization assay, cells (or lenses) were then resuspended in 1 ml of lysis buffer (5 mM Tris base, 2 mM EDTA, 2 mM EGTA, 0.5 mM diisopropylfluorophosphate, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and 200 μM leupeptin). After a 10-min incubation at 4°C, the swollen cells were disrupted by repeated passage (25–30 times) through a 25-gauge needle, and the resulting cell lysates were brought to isotonicity by addition of 100 μl of a 10× PBS stock solution. Alternatively, cells were disrupted under isotonic conditions in either PBS or L-15 medium, with identical results. 20% Triton X-100 was added to a final concentration of 1% (wt/wt), and the lysates were incubated for 30 min at 4°C with occasional resuspension by vortexing. Half of the sample (575 μl) was then reserved at 4°C (total cell lysate) whereas the remainder was subjected to centrifugation at 100,000 *g* for 50 min at 4°C. The supernatant fraction (Triton-soluble lysate) was carefully removed, brought to 1.2% SDS, and boiled for 3 min. When cool, the sample was diluted with 900 μl of immunoprecipitation buffer (Musil et al., 1990*a*) supplemented with 0.5 M sucrose, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and enough Triton X-100 to bring the final detergent concentration to 2% Triton X-100 and 0.4% SDS. The total cell lysate sample was processed identically. The material pelleted at 100,000 *g* (Triton-insoluble lysate) was resuspended to 575 μl with immunoprecipitation buffer supplemented with 2 mM PMSF and 1.2%

SDS and then boiled for 3 min, after which it was diluted with Triton-containing immunoprecipitation buffer as described for the Triton-soluble supernatant. All three fractions were then immunoprecipitated with affinity-purified antibodies to connexin43 (252–271) using our standard protocol (see above). In certain experiments the buffer conditions, amount of Triton, temperature, and/or time of solubilization were altered as specified in Table I.

For solubilization in sarcosine, NRK cells or embryonic chick lenses were disrupted in 5 mM Tris base, 0.5 mM diisopropylfluorophosphate, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and 200 μM leupeptin, pH 10.0, and incubated for 10 min at 4°C. *N*-laurylsarcosine was then added to a final concentration of 0.3%, after which the lysates were incubated for 10–15 min at 25°C with occasional vortexing. The samples were then centrifuged at 100,000 *g* and processed as described for Triton-solubilized cultures.

In Situ Extraction of NRK Cells with Triton

NRK cell cultures grown on uncoated 35-mm tissue culture dishes were rinsed three times at 4°C with incubation buffer (8.0 g NaCl, 0.4 g KCl, 0.09 g $Na_2HPO_4 \cdot 7H_2O$, 0.047 g KH_2PO_4, 0.097 g $MgSO_4$, 0.4 g $CaCl_2.2H_2O$, and 4.76 g Hepes per liter of H_2O; pH 7.5). The cells were then equilibrated in the same buffer for 20 min at 14°C, after which the buffer was removed and replaced with 1 ml of incubation buffer supplemented with 0.5 mM diisopropylfluorophosphate, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and 200 μM leupeptin in either the absence (mock-extracted cells) or presence (Triton-extracted cells) of 1% Triton X-100. The cultures were gently rotated on an orbital shaker at 14°C for 30 min, after which the extraction buffer was carefully removed and the cultures were rinsed five times with incubation buffer containing 0.5 mM diisopropylfluorophosphate and 2 mM PMSF. In the case of Triton-extracted cultures, this buffer contained 1% Triton for the first three rinses. Care was taken not to disrupt the cell monolayer during the wash steps.

For biochemical analysis of connexin43 remaining with the monolayer, 300 μl of lysis buffer (described earlier) containing 0.6% SDS was added to the tissue culture dish, and the lysates boiled for 3 min before immunoprecipitation of connexin43. For immunofluorescent localization of connexin43, the extracted or mock-extracted cells were fixed for 1 h at room temperatures 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, incubated overnight at 4°C with a 1:100 dilution of affinity-purified antibodies to connexin43 (252–271), and incubated with 1:500 rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) as previously described (Musil et al., 1990*b*). The cultures were photographed on an Axioscope microscope (Carl Zeiss, Inc., Oberkochen, Germany) fitted with the appropriate filters.

Electron Microscopy

NRK cell cultures either mock extracted in the absence of Triton or extracted with Triton for 30 min (at 14°C or 4°C) were fixed by addition of 2.5% glutaraldehyde and 1% tannic acid in 0.1 M cacodylate (pH 7.4) directly to the tissue culture dish. The specimens were then postfixed in 1% OsO_4 and stained with 1% uranyl acetate in situ, after which cell sheets were scraped from the tissue culture dish prior to dehydration and embedding in epoxy resins. Thin sections were cut and observed using a JEOL 100CX electron microscope operating at 60 kV.

Cell Surface Biotinylation

Biotinylation of cell monolayers was conducted using a modification of the procedure described by Le Bivic et al. (1989 and 1990*b*). Metabolically labeled 60 mm cell cultures were rinsed three times at 4°C with PBS containing 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS+) and incubated on ice for 10 min in the same buffer. After rinsing the cultures twice with PBS+, cell-surface biotinylation was initiated by addition of 2 ml of 0.5 mg/ml NHS-LC-biotin in PBS+ to each dish and the cultures were then incubated for 30 min with gentle agitation. The reaction was quenched by rinsing the cultures five times with L-15 medium supplemented with 15 mM glycine; there was a 10-min incubation in L-15/glycine between the third and fourth washes. All manipulations were conducted on ice in a room at 4°C. In some cases, the biotinylated monolayers were immediately lysed with 500 ul of lysis buffer (described earlier) supplemented with 0.6% SDS and 10 mM glycine and boiled for 3 min, or subjected to our standard Triton solubilization assay in the presence of 10 mM glycine. Alternatively, the cells were chased for 1–3 h at 37°C in L-15 medium supplemented with 10 mM Hepes, 10% FCS, and 0.5 mM methionine before lysis. Connexin43 was then immunoprecipitated from all samples using affinity-purified antibodies to connexin43 (252–271) and protein A-Sepharose beads as previously described. Immunoprecipitated connexin43 was eluted from the beads by boiling them for 4 min in 40 μl of immunoprecipitation buffer containing 10% SDS and 2 mM PMSF. One fifteenth of the eluted connexin43 was added to SDS-PAGE sample buffer and reserved as a sample of total cellular connexin43. The remainder was diluted with immunoprecipitation buffer supplemented with 0.5% BSA, 10 mM *N*-ethylmaleimide, 2 mM PMSF, and enough Triton X-100 to bring the final detergent concentration to 1% Triton and 0.2% SDS. Biotinylated connexin43 was recovered from this sample by a second round of precipitation with avidin-agarose (35 μl, 50% slurry), after which the beads were washed as described above following immunoprecipitation of connexin43 with protein A-Sepharose. The biotinylated connexin43 was eluted from the avidin-agarose by boiling in SDS-PAGE sample buffer for 5 min and analyzed (along with the total cellular connexin43 sample) by SDS-PAGE. In control experiments, connexin43 was immunoprecipitated from [^{35}S]methionine-labeled, unbiotinylated NRK cells and then subjected to a second round of precipitation with protein A-Sepharose instead of avidin-agarose. When analyzed by SDS-PAGE, the amount and phosphorylation state of this material appeared identical to [^{35}S]connexin43 that had been subjected to only a single round of precipitation, indicating that artifactual dephosphorylation or degradation of connexin43 did not occur during the double precipitation procedure (not shown).

Inhibition of Junctional Permeability with Heptanol or 100% CO_2

Heptanol (Fisher Scientific, Fair Lawn, NJ) was diluted fresh daily 1:4 in ethanol and used at a final concentration of 3.5 mM as previously described (Musil et al., 1990*b*). For cytoplasmic acidification of NRK cells, humidified 100% CO_2 was bubbled through MEM formulated with Earle's salts (plus L-glutamine and 1% FCS) for 10 min to reduce the pH of the medium to 6.0. NRK cultures were then immediately incubated in this medium for 10–15 min at 37°C (Schuetze and Goodenough, 1982).

Results

Differential Triton X-100 Solubility of Phosphorylated and Nonphosphorylated Forms of Connexin43

Our previous studies characterized the biosynthesis of connexin43 in NRK cells, a gap junctional communication-competent cell line that assembles connexin43 into large gap junctional plaques (Musil et al., 1990*b*). When NRK cultures are metabolically labeled with [^{35}S]methionine for 5 h, lysed, and immunoprecipitated with antibodies affinity purified from rabbit anti-connexin43 (252–271) serum (Beyer et al., 1989), three [^{35}S]methionine-labeled connexin43-related species are obtained (Fig. 1 *A*, lane *1*). The fastest migrating of these bands (M_r, ~42,000) has been shown to represent newly synthesized connexin43 and comigrates with connexin43 translated in a cell-free reticulocyte lysate system (Musil et al., 1990*a*). Since this species is not metabolically labeled with ^{32}P (Fig. 1 *A*, lane *2*) and is insensitive to treatment with alkaline phosphatase (Musil et al., 1990*a* and *b*), we refer to it as connexin43-NP (not phosphorylated).

Pulse–chase studies have determined that connexin43-NP is posttranslationally modified in NRK cells to a 44-kD and then to a 46-kD species. Both of these bands incorporate ^{32}P (Fig. 1 *A*, lane *2*) and are converted to the connexin43-NP form by alkaline phosphatase treatment (Musil et al., 1990*a* and *b*). This finding demonstrates that the posttranslational shift in the apparent molecular weight of connexin43 is due exclusively to the addition of phosphate. The lower and upper bands of the phosphorylated connexin43 doublet have been designated connexin43-P_1 and connexin-43P_2, respectively (Musil et al., 1990*b*). A similar pattern of

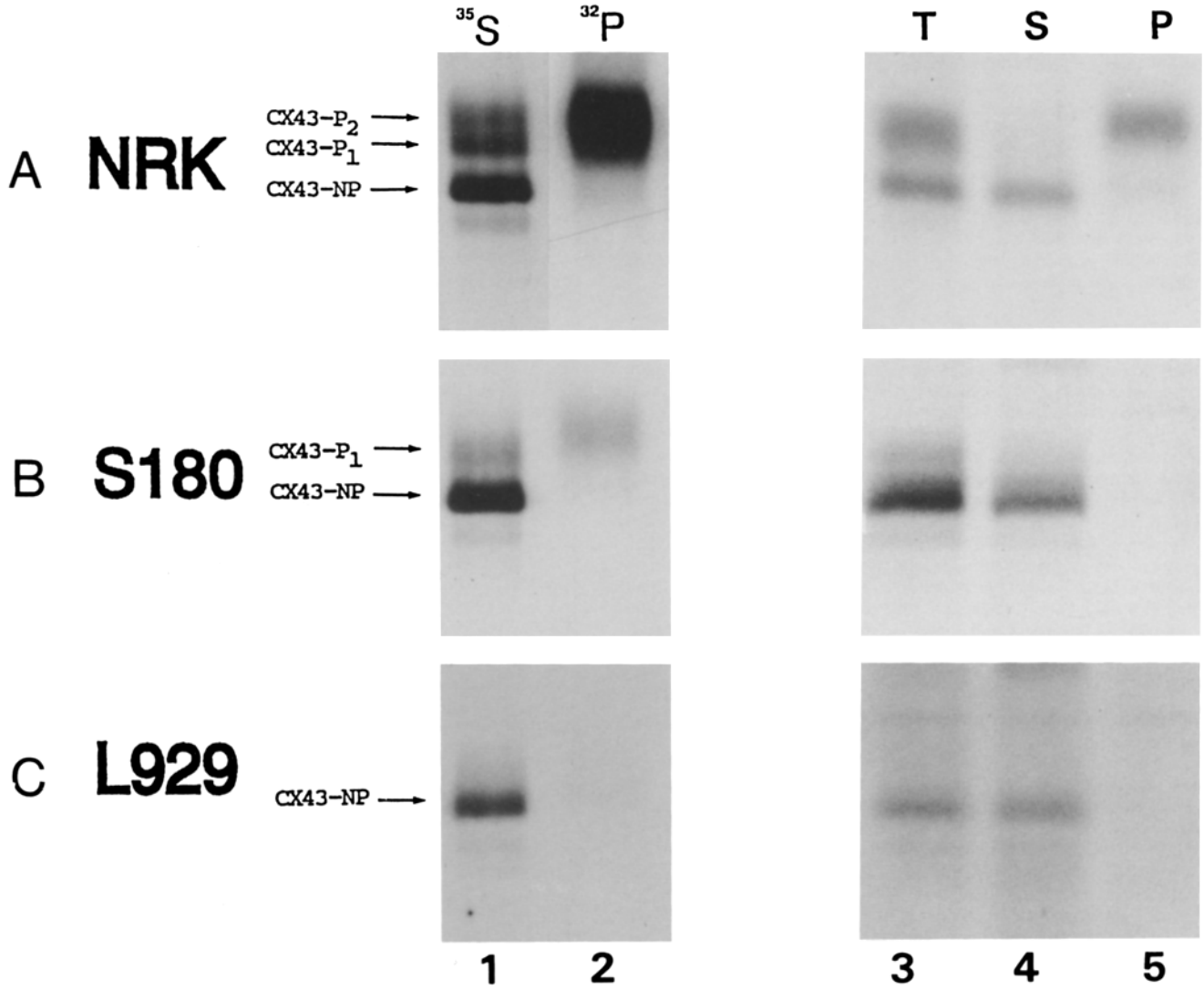


Figure 1. Solubility of phosphorylated and nonphosphorylated forms of connexin43 in Triton X-100. Confluent cultures of communication-competent NRK (*A*) or communication-deficient S180 (*B*) or L929 (*C*) cells were metabolically labeled with either [^{35}S]methionine (lanes *1*, and *3–5*) or [^{32}P]O_4 (lane *2*) for 5 h. In lanes *1* and *2*, the cells were immediately lysed and immunoprecipitated with affinity-purified antibodies to connexin43 (252–271). Lanes *3–5* are from a separate experiment in which cells were homogenized and incubated with Triton X-100 under our standard solubilization conditions (1% Triton in PBS, 30 min, 4°C). Half of the Triton-treated lysate was reserved on ice (total cellular lysate), whereas the remainder was subjected to centrifugation at 100,000 *g* for 50 min. Connexin43 was then immunoprecipitated from equal amounts of the total cellular lysate (*T*, lane *3*), Triton-soluble supernatant (*S*, lane *4*), or Triton-insoluble pellet (*P*, lane *5*) fractions. Connexin43-P_1 and -P_2 immunoprecipitated from the insoluble fraction routinely migrated slightly slower than that recovered from the total cellular lysate because of a difference in the Triton content of the two fractions during the SDS denaturation step (*A*, lanes *3* vs. *5*; see Materials and Methods).

connexin43 biosynthesis and phosphorylation has been observed in other communication-competent cell types (Musil et al., 1990*a* and *b*). In addition to these three major forms of connexin43, a barely detectable species that migrates slightly slower than connexin43-NP and that can be metabolically labeled with ^{32}P was occasionally observed; this band may represent partially dephosphorylated connexin43 or a minor additional form of connexin43 and will not be considered further.

The phosphorylated and nonphosphorylated forms of connexin43 displayed different solubilities in the nonionic detergent Triton X-100 (Fig. 1 *A*). 3-d-old, newly confluent monolayers of NRK cells were metabolically labeled for 5 h with [^{35}S]methionine, after which the cells were scraped from the tissue culture dish and disrupted by repeated passage through a 25-gauge needle. The cell lysates were then incubated under isotonic conditions with 1% (wt/wt, final concentration) Triton X-100 for 30 min at 4°C. Half of the lysate was stored on ice to serve as the total connexin43 sample; the remainder was subjected to centrifugation at 100,000 *g* for 50 min to separate the Triton-soluble from the Triton-insoluble material. Immunoprecipitation of connexin43 from the total (Fig. 1 *A*, lane *3*), the supernatant (Triton-soluble, lane *4*), and pellet (Triton-insoluble, lane *5*) fractions revealed that ~80–90% of connexin43-NP was solubilized by 1% Triton X-100. In contrast, connexin43-P_2 was quantitatively recovered in the pellet fraction and remained insoluble upon reextraction with 1% Triton (not shown). Connexin43-P_1 was not well resolved from connexin43-P_2 after Triton treatment and displayed an inter-

mediate and somewhat variable sensitivity to Triton; most of the connexin-43-P_1 fractionated with connexin43-P_2 (Fig. 1 *A*, lane *5*), but some was detectable in the Triton-soluble supernatant (lane *4*). Similar results were obtained with 2–5-day-old cultures, indicating that cell density and/or age were not critical factors in determining the resistance of connexin43 to Triton. Connexin43 from S180L cells, another communication-competent cell line that forms large gap junctional plaques (Mege et al., 1988; Musil et al., 1990*b*), displayed a pattern of Triton X-100 solubility identical to that observed in NRK cells (data not shown).

Connexin43 is also synthesized by certain communication-defective cell lines, including mouse sarcoma 180 (S180) and fibroblastic L929 cells (Musil et al., 1990*b*). These cells have been shown to be severely deficient (S180 cells) or completely lacking (L929 cells) in morphologically or physiologically recognizable gap junctions (Furshpan and Potter, 1968; Mege et al., 1988; Larson et al., 1990). S180 cells process connexin43 to the connexin43-P_1 form to a lesser extent than do NRK cells (compare Fig. 1 *B*, lane *1* with Fig. 1 *A*, lane *1*) and lack all but a trace of connexin43-P_2, which was detectable only as a fraction of ^{32}P-labeled connexin43 (Fig. 1 *B*, lane *2*). L929 cells contain neither connexin43-P_1 nor-P_2 (Fig. 1 *C*, lanes *1* and *2*; see also Musil et al., 1990*b*). When S180 (Fig. 1 *B*, lanes *3–5*) or L929 (Fig. 1 *C*, lanes *3–5*) cultures were subjected to the Triton solubility assay exactly as described for NRK cells, connexin43-NP was immunoprecipitated only from the soluble fraction; recovery ranged from 66–90%. Connexin43 is as metabolically stable in S180 and L929 cells as in NRK cells (Musil et al., 1990*b*), ruling out the possibility that communication-deficient cells degraded connexin43 at a rate faster than the rate of conversion to the Triton-insoluble form. Together, these results suggest that solubility in Triton X-100 is correlated with the phosphorylation state of connexin43 rather than with its cell of origin.

Table I summarizes the solubility properties of connexin43 in Triton X-100. The differential solubility of phosphorylated and nonphosphorylated forms of connexin43 in NRK cells depicted in Fig. 1 *A* were obtained with Triton X-100 concentrations ranging from 0.04–4.0% and was unaffected by the presence of 2-mercaptoethanol, divalent cations, or prolongation of the solubilization period from 30 min to 24 h. Thus, the resistance of connexin43-P_2 to Triton was not due to disulfide bonds or to Ca^{2+}- or Mg^{2+}-dependent polymerization, possibilities raised by earlier studies (Zampighi and Robertson, 1973; Manjunath and Page, 1986). The addition of high salt concentration (0.5 M NaCl) to the solubilization buffer did, however, render connexin43-P_2 partially soluble in 1% Triton X-100, a fact suggesting that ionic interactions may be involved in maintaining connexin43-P_2 in a Triton-insoluble state. When the temperature of the isotonic Triton treatment was raised from 4°C to 25°C, connexin43-P_2 remained insoluble in 0.04% Triton but was partially solubilized by 1% Triton X-100, probably reflecting increased detergent activity at higher temperatures. Neither phosphorylated nor nonphosphorylated forms of connexin43 were solubilized by levels of Triton X-100 below the critical micellar concentration under any of the conditions tested.

Table I. Detergent Solubility of Connexin43 in NRK Cells

All forms of connexin43 insoluble
0.005% Triton X-100; 4°C or 25°C (<cmc)
0.005% Triton X-100 in PBS + 500 mM NaCl
0.005% LDAO (<cmc)
5 mM Tris, pH 10.0; 30 min, 25°C
Connexin43-NP soluble, connexin43-P_2 insoluble
0.04%–4.0% Triton X-100 (>cmc)
1.0% Triton X-100; 0.5–24 h, 4°C
1.0% Triton X-100:
± 5% 2-mercaptoethanol
± 1 mM Ca^{2+}, Mg^{2+}
± 5 mM EDTA, EGTA
0.04% Triton X-100; 30 min, 25°C
Connexin43-NP soluble, connexin43-P_2 partially soluble
1.0% Triton X-100; 30 min, 25°C
1.0% Triton X-100 in PBS + 500 mM NaCl
0.3% deoxycholate ± 10 mM *N*-ethylmaleimide*
All forms of connexin43 soluble
0.3% *N*-lauryl sarcosine in 5 mM Tris, pH 10.0; 10 min, 25°C
0.07% LDAO (>cmc)

Three-d-old, newly confluent cultures of NRK cells were metabolically labeled for 5 h with [^{35}S]methionine and then homogenized as described in Materials and Methods. The cell lysates were subsequently incubated under the conditions specified above; unless noted otherwise, all reactions were conducted in PBS, 2 mM EDTA, 2 mM EGTA, pH 7.4, for 30 min at 4°C. The lysates were then subjected to centrifugation at 100,000 *g* for 50 min at 4°C, after which connexin43 was immunoprecipitated from the soluble supernatant and insoluble pellet fractions and analyzed by SDS-PAGE.
* Connexin43-P_2 was more soluble in 0.3% deoxycholate in PBS, pH 7.4, than in 5 mM Tris, pH 10.0.
LDAO, lauryldimethylamine oxide; cmc, critical micellar concentration.

Solubility of Connexin43 in Other Detergents

Relative insolubility in the ionic detergent *N*-lauryl sarcosine is the basis for several procedures for isolation of gap junctions from whole organs such as heart (Kensler and Goodenough, 1980; Manjunath et al., 1984) and liver (Goodenough and Stoeckenius, 1972; Hertzberg and Gilula, 1979). It was therefore of interest to examine the behavior of connexin43 from NRK cells in this detergent (Fig. 2 *A*). Lysates were prepared from [^{35}S]methionine-labeled NRK cultures in 5 mM Tris, pH 10.0, and incubated with 0.3% *N*-lauryl sarcosine for 10 min at room temperature, solubilization conditions identical to those used during the preparation of connexin43-containing gap junctions from rat heart (Kensler and Goodenough, 1980; Manjunath et al., 1984). After centrifugation at 100,000 *g* for 50 min, >97% of the total (both phosphorylated and nonphosphorylated) connexin43 recovered by immunoprecipitation was in the soluble fraction (Fig. 2 *A*, lane 2). Overexposure of the fluorograph revealed a small amount of connexin43-P_2 in the insoluble fraction; whether this material represents bona fide sarcosine-insoluble connexin43 or is due to nonspecific trapping of soluble connexin43 in the pellet is unknown. The ability to solubilize connexin43-P_2 was not unique to sarcosine since identical results were obtained with the nonionic detergent lauryldimethylamine oxide. Phosphorylated forms of connexin43 were also partially soluble in deoxycholate, the extent of solubilization being dependent on the composition of the lysis buffer (Table I).

The lack of resistance of connexin43 in NRK cells to *N*-lauryl sarcosine appeared to be at variance with the reported sarcosine-insolubility of connexin43 in rat heart (Kensler and Goodenough, 1980; Manjunath et al., 1984). A potential explanation for this discrepancy was that connexin43 is somehow organized or assembled differently in tissue culture

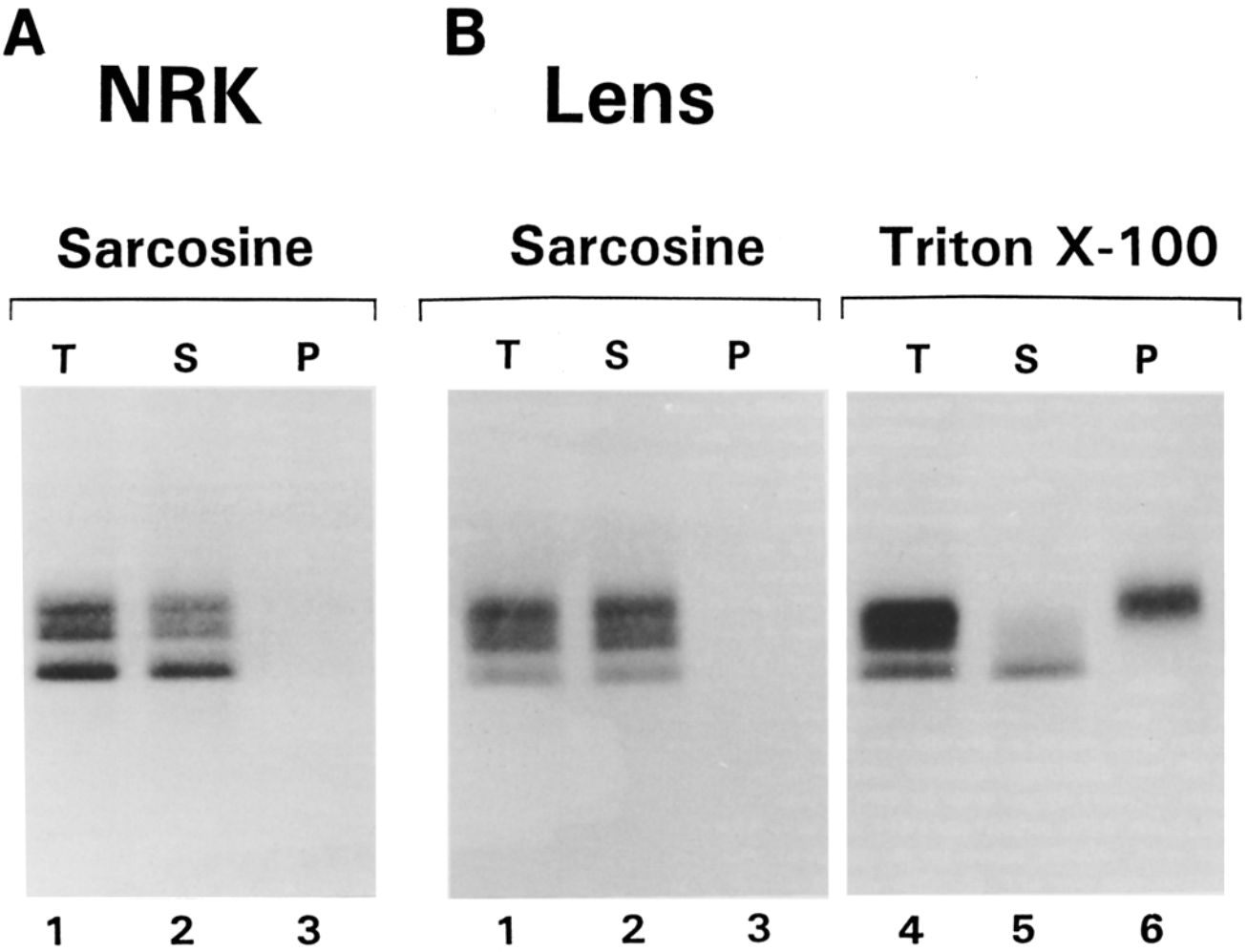


Figure 2. Comparison of the detergent solubility of connexin43 from NRK cells and from chick lenses. NRK cultures (*A*) or intact embryonic chick lenses (*B*) were metabolically labeled for 5 h with [^{35}S]methionine, homogenized, and incubated either with 0.3% *N*-lauryl sarcosine in 5 mM Tris (pH 10.0) for 10 min at 25°C (*A* and *B*, lanes *1–3*), or with 1% Triton X-100 under our standard solubilization conditions (PBS pH 7.4, 30 min, 4°C) (*B*, lanes *4–6*). Half of the cell lysate was then centrifuged at 100,000 *g* for 50 min, after which connexin43 was immunoprecipitated from equal amounts of the total cellular lysate (lanes marked *T*), detergent-soluble supernatant (lanes marked *S*), or detergent-insoluble pellet (lanes marked *P*) fractions.

cells than in whole organs. To test this possibility, we examined the detergent solubility of connexin43 in embryonic (d 10) chick lens. Our previous studies demonstrated that epithelial cells from this organ synthesize connexin43 and incorporate it into large, communication-competent gap junctional plaques (Musil et al., 1990*a*). Intact lenses (stripped of associated ciliary epithelium) were incubated with [^{35}S]-methionine for 5 h. The labeled lenses were then disrupted at 4°C in hypotonic Tris buffer and incubated with 1% Triton X-100 (in PBS, at 4°C) or 0.3% *N*-lauryl sarcosine (in 5 mM Tris, pH 10, at 25°C), exactly as described for NRK cell lysates. As shown in Figure 2 *B*, the pattern of solubility of [^{35}S]methionine-labeled connexin43 in chick lens epithelium was similar to that obtained in NRK cells in both detergents (compare Fig. 1 *A*, lanes *3–5* with Fig. 2 *B*, lanes *4–6*; Fig. 2 *A*, lanes *1–3* with Fig. 2 *B*, lanes *1–3*). By these criteria, then, the solubility properties of connexin43 from an organ (lens) are similar to those of connexin43 from tissue culture cells (NRK cells). Possible explanations for the recovery of sarcosine-insoluble gap junctions from heart are considered in the Discussion.

Time Course of Acquisition of Triton X-100 Resistance by Connexin43

In communication-competent cell types thus far examined, connexin43-NP is the kinetic precursor of connexin43-P_2. It therefore seemed likely that connexin43 is synthesized in a Triton-soluble form that acquires Triton resistance as it matures. This possibility was confirmed in a pulse–chase experiment (Fig. 3). Confluent monolayers of NRK cells were labeled with [^{35}S]methionine for 40 min and then chased in the presence of an excess of unlabeled methionine for ≤6 h before cell lysis and assessment of connexin43 Triton solubility under our standard assay conditions. As expected, [^{35}S]methionine–connexin43 examined immediately after the pulse period was entirely in the connexin43-NP form and was quantitatively solubilized by 1% Triton (Fig. 3, lanes *1–3*). After 1 h of chase, about one third of [^{35}S]methionine–connexin43 had been phosphorylated to connexin43-P_1, with no detectable conversion to connexin43-P_2 (Fig. 3, lane *4*). This connexin43-P_1 was recovered in both the Triton-soluble (Fig. 3, lane *5*) and -insoluble (Fig. 3, lane

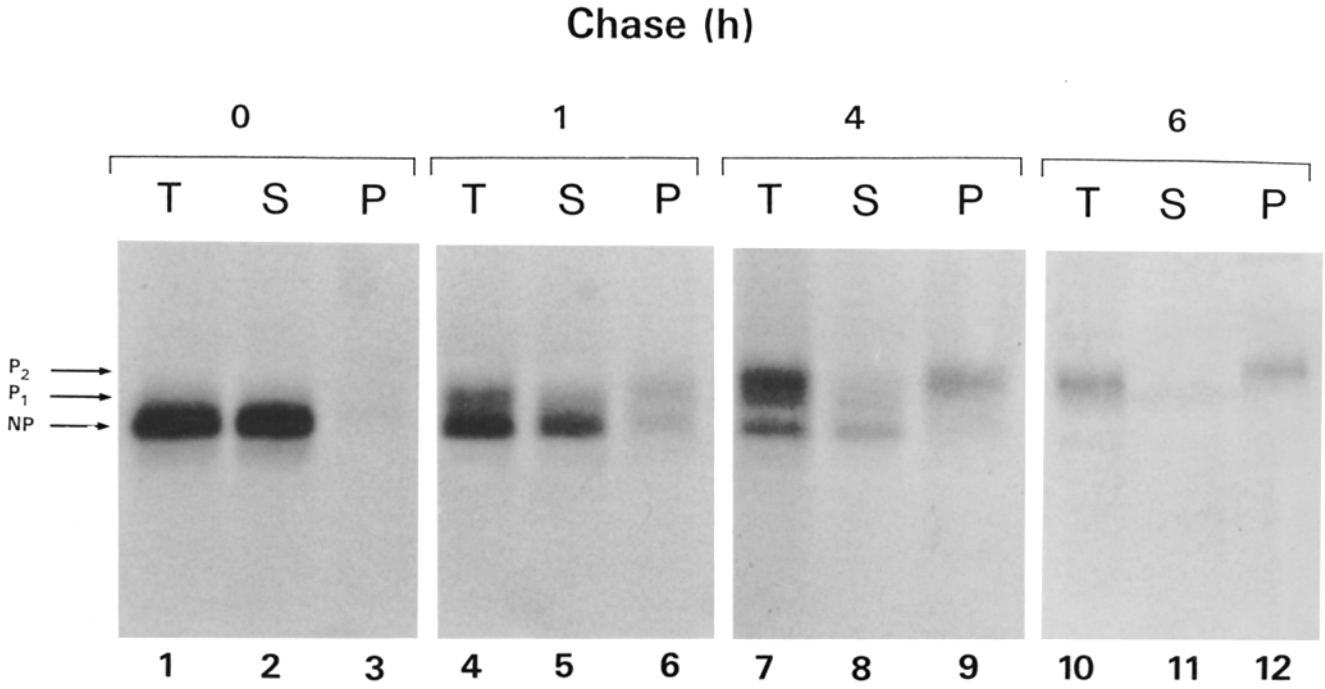


Figure 3. Pulse-chase analysis of the Triton solubility of connexin43 in NRK cells. Confluent monolayers of NRK cells were metabolically labeled with [^{35}S]methionine for 40 min and chased for 0, 1, 4, or 6 h. The cultures were then homogenized and subjected to the standard Triton solubilization assay (1% Triton X-100 in PBS, 30 min, 4°C), after which equal amounts of the various fractions were immunoprecipitated with affinity purified anti-connexin43 (252–271) antibodies. Lanes marked *T* (*1*, *4*, *7*, and *10*), total cellular connexin43; lanes marked *S* (*2*, *5*, *8*, and *11*), Triton-soluble connexin43; lanes marked *P* (*3*, *6*, *9*, and *12*), Triton-insoluble connexin43. Lanes *7–12* were exposed longer than the other lanes to double the intensity of the connexin43 signal. Connexin43-P_1 (lanes *6* and *9*) and connexin43-P_2 (lanes *9* and *12*), immunoprecipitated from the insoluble fraction, routinely migrated slightly slower than that recovered from the corresponding total cellular lysate due to a difference in the Triton content of the two fractions during the SDS denaturation step (see Materials and Methods).

6) fractions, similar to the distribution of connexin43-P_1 in NRK cells labeled to steady state (Fig. 1 *A*, lanes *3–5*). Connexin43-NP remained predominantly Triton-soluble, although a minor fraction (~20%) was consistently recovered in the Triton-insoluble pellet beginning about this time. At 4 h of chase, 66% of the [^{35}S]methionine–connexin43 synthesized during the pulse period had been degraded, a finding consistent with the 2–2.5 h half-life of connexin43 in NRK and other cell types (Musil et al., 1990*b*). The remaining [^{35}S]methionine–connexin43 was largely processed to the P_2 form and was quantitatively resistant to Triton solubilization, whereas the residual [^{35}S]methionine–connexin43-NP detectable at this time was mainly Triton soluble (Fig. 3, lanes *7–9*). Low levels of Triton-insoluble connexin43-NP as well as Triton-soluble connexin43-P_1 were, however, still present.

The rapid turnover rate of connexin43 thwarted attempts to determine whether Triton-insoluble connexin43-NP served as a precursor to connexin43-P_2 or was degraded without being phosphorylated; similarly, the fate of Triton-soluble connexin43-P_1 was unknown. It was thus not possible to conclude from these data whether most of the connexin43-NP was phosphorylated to the connexin43-P_1 form before or after becoming Triton insoluble. In either case, connexin43-P_2 was completely resistant to Triton and appeared to remain so throughout the lifetime of the protein (Fig. 3, lanes *10–12*). Taken together, these results indicate a strong temporal correlation between processing of connexin43 to the P_2 form and the acquisition of insolubility in Triton X-100. However, whether these two events normally occur in rapid succession or simultaneously is not known.

Morphological Localization of the Triton-insoluble Pool of Connexin43

We exploited the Triton resistance of connexin43-P_2 to determine the intracellular distribution of the various forms of connexin43 using an in situ extraction procedure (Fig. 4). Monolayer cultures of NRK cells were washed three times and then extracted by addition of 1% Triton X-100–containing isotonic buffer directly to the tissue culture dish. After a 30-min incubation, the cultures were carefully rinsed five times to remove solubilized cellular components. The connexin43 remaining with the extracted monolayer was analyzed either biochemically by immunoprecipitation or morphologically by immunofluorescence using affinity-purified antibodies to connexin43 (252–271). Preliminary studies demonstrated inefficient solubilization of connexin43-NP after in situ extraction of [^{35}S]methionine-labeled NRK cells at 4°C for ≤2 h (data not shown). When the extraction temperature was raised from 4°C to 14°C, however, 75% of [^{35}S]methionine–connexin43-NP was released from the cells within 30 min (Fig. 4 *A*, *inset*, lane *1* vs. *2*); the proportion released was comparable to the fraction of connexin43-NP solubilized from disrupted NRK cell lysates in the standard Triton solubilization assay (Fig. 1 *A*, lanes *3–5*). In contrast,

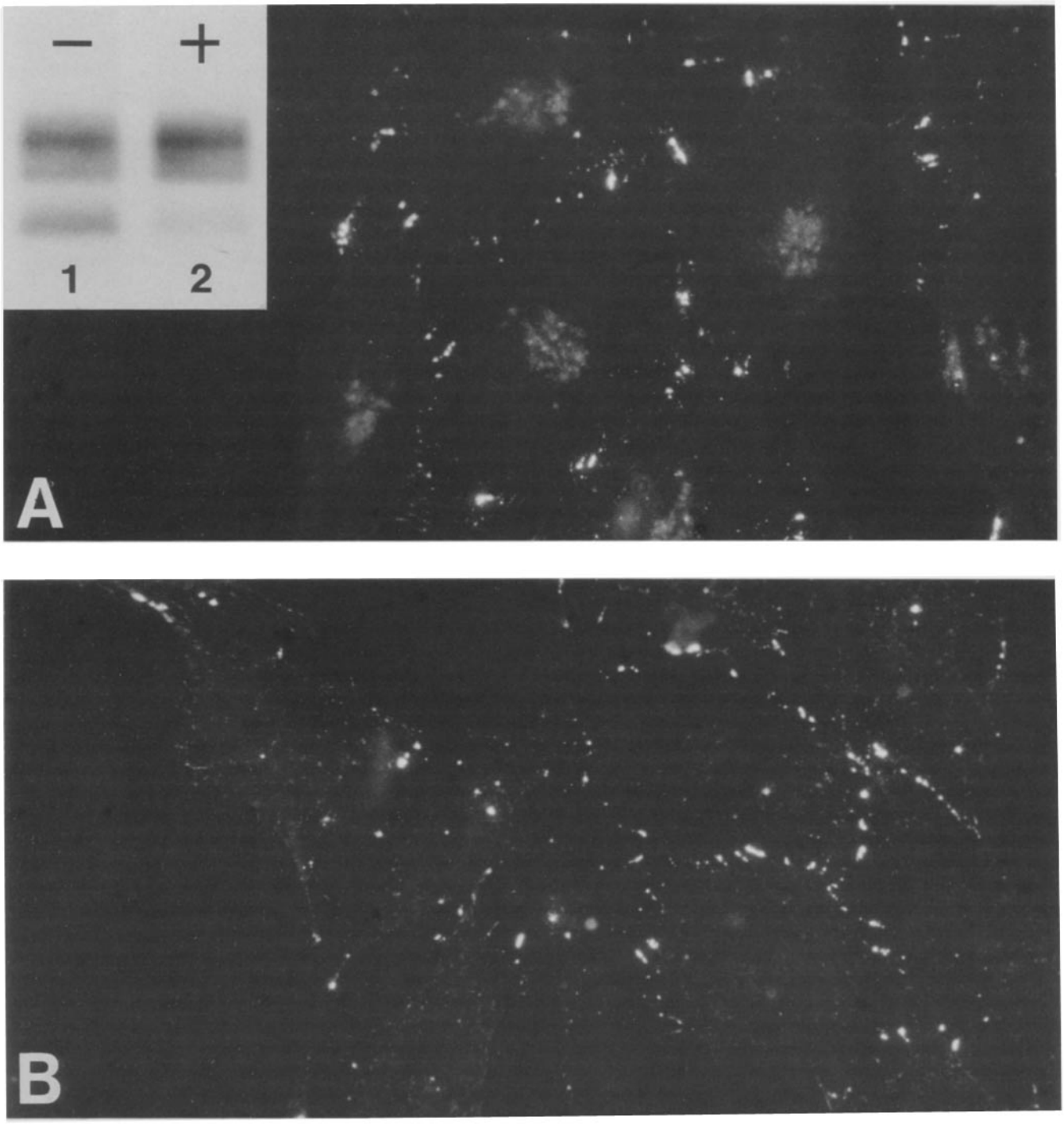
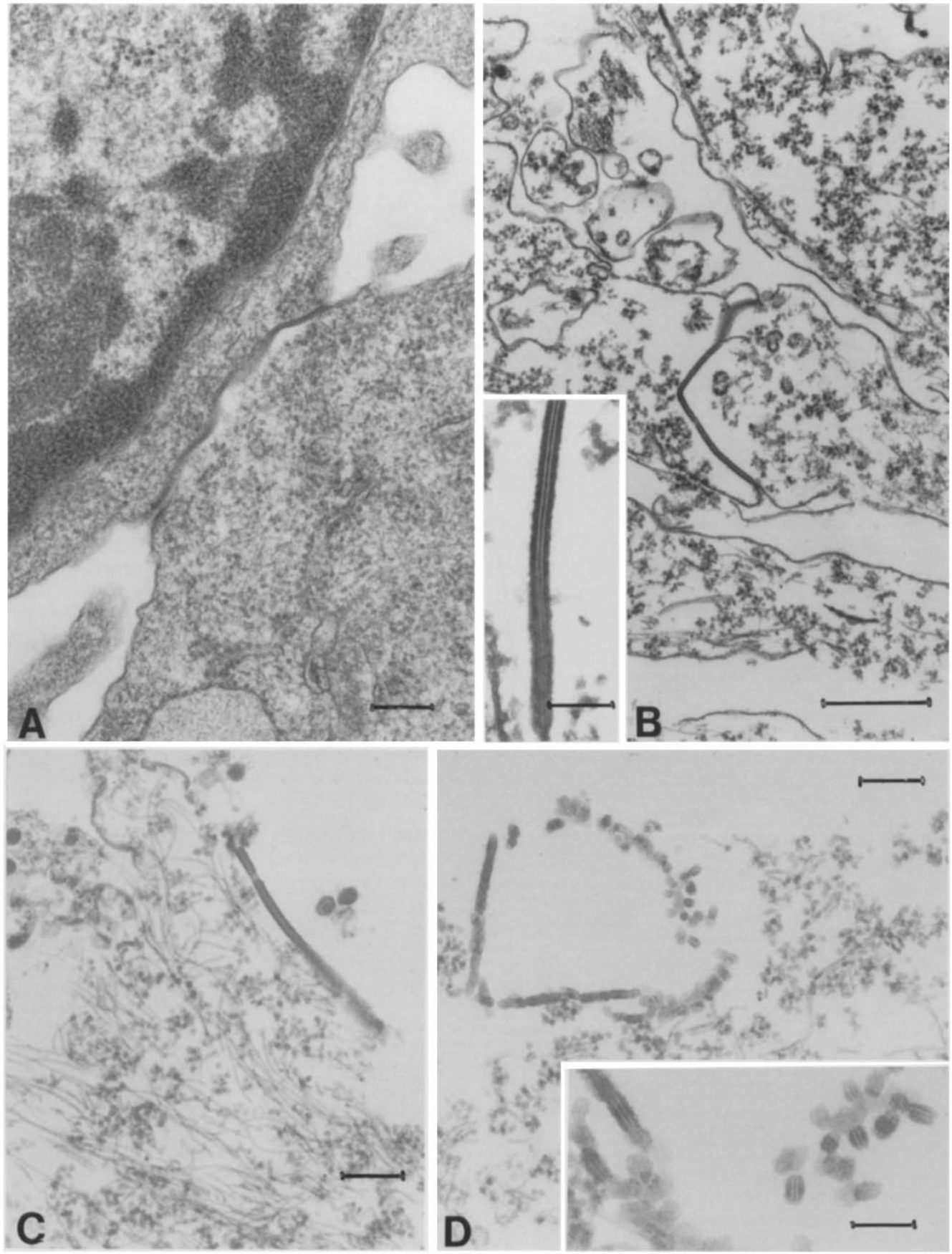


Figure 4. In situ extraction of Triton-soluble connexin43-NP in NRK cells. NRK monolayers were incubated at 14°C for 30 min under isotonic conditions in either the absence (*A*) or presence (*B*) of 1% Triton X-100. After extensive washing to remove solubilized material, the cultures were fixed and processed for immunofluorescence using anti-connexin43 (252–271) antibodies followed by rhodamine-labeled goat anti-rabbit IgG. (*Inset*) NRK cultures metabolically labeled with [^{35}S]methionine for 5 h were extracted either with (lane 2) or without (lane *1*) 1% Triton exactly as described for unlabeled cells and then immunoprecipitated with anti-connexin43 (252–271) antibodies. Note that [^{35}S]methionine–connexin43-P_2 is resistant to Triton solubilization and appears to be localized to brightly staining maculae at cell–cell interfaces.

Figure 5. Thin-section electron microscopy of gap junctions in Triton-extracted and mock-extracted NRK cultures. (*A*) Low-magnification photomicrograph of a gap junction between two mock-extracted NRK cells. Bar, 0.2 μm. (*B*) Gap junction in an NRK culture subjected to in situ extraction with 1% Triton at 4°C. Bar, 0.5 μm. (*Inset*) Higher-magnification view of the same junction. Bar, 0.1 μm. (*C* and *D*) Gap junctions in NRK cells extracted in situ with 1% Triton at 14°C. Some junctions appear morphologically intact (*C*), whereas others have partially disassembled into short, double-membrane structures (*D*). Bar, 0.2 μm. (*D, inset*) Higher magnification of *D*, showing pentalaminar structure of the gap junction fragments. Bar, 0.1 μm.


A
B
C
D

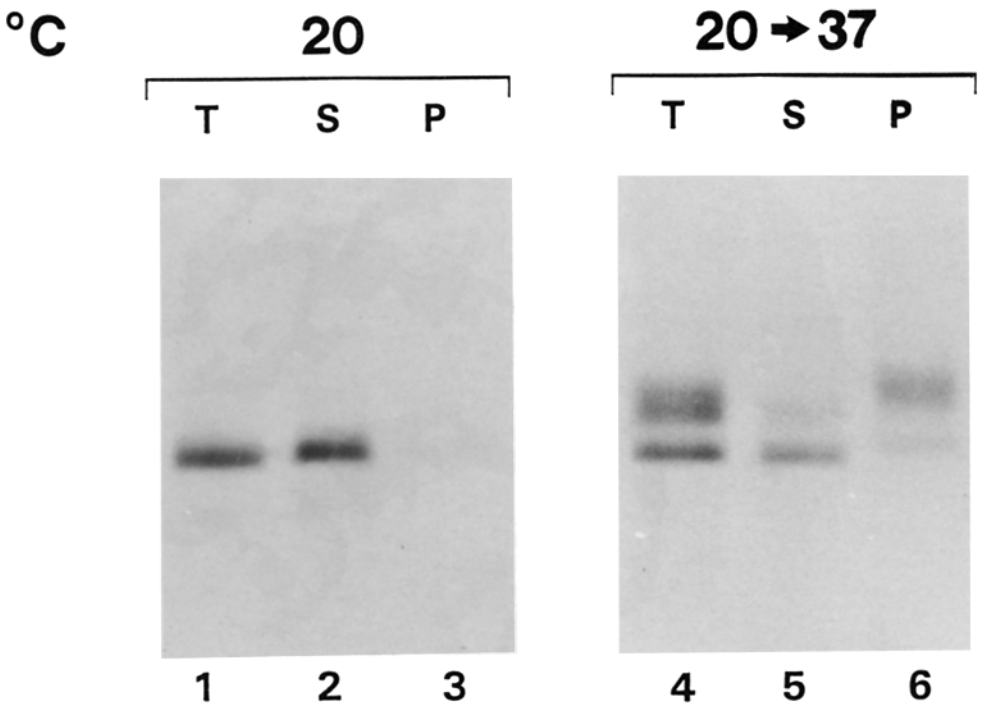


Figure 6. Inhibition of connexin43 posttranslational processing at 20°C. Duplicate cultures of confluent NRK cells were metabolically labeled with [^{35}S]methionine at 20°C for 4 h. The monolayers were then subjected to our standard Triton X-100 solubility assay (1% Triton in PBS, 30 min, 4°C), either immediately after labeling at 20°C (lanes *1–3*) or after a 2-h chase at 37°C in L-15 medium supplemented with 10 mM HEPES, 10% FCS, and 0.5 mM methionine (lanes *4–6*). Connexin43 was immunoprecipitated from equal volumes of the total cell lysate (lanes *1* and *4*), Triton-soluble (lanes *2* and *5*), and Triton-insoluble (lanes *3* and *6*) fractions.

[^{35}S]methionine–connexin43-P_2 was resistant to this treatment and was quantitatively recovered with the extracted monolayer (Fig. 4 *A*, *inset*). Immunohistochemical studies revealed that control monolayers incubated with only buffer before fixation (Fig. 4 *A*) showed a pattern of macular immunoreactivity at cell–cell interfaces typical of gap junctions (Beyer et al., 1989). In addition, intracellular structures morphologically similar to the Golgi apparatus were labeled, consistent with our previous results with these cells (Musil et al., 1990*b*). In situ extraction of NRK cells with 1% Triton X-100 at 14°C before fixation abolished this Golgi-like immunofluorescent staining, leaving the macular signal at cell–cell interfaces (Fig. 4 *B*). A fine, speckled pattern of staining distributed throughout the extracted cells was also visible. This diffuse immunoreactivity was obtained with preimmune serum as well, indicating that it does not represent connexin43. In contrast, the plaque-like signal at cell–cell interfaces was not detectable when preimmune serum was substituted for the antibodies to connexin43 (not shown).

To confirm that the Triton-resistant maculae were gap junctional plaques, Triton-extracted and unextracted NRK cells were examined by thin-section EM (Fig. 5). Mock-extracted cells not exposed to Triton contained pentalaminar structures at cell–cell interfaces that are diagnostic of gap junctions (Fig. 5 *A*). These profiles were maintained when NRK cells were extracted with 1% Triton at 4°C before in situ fixation, conditions under which connexin43-P_2 is quantitatively recovered with the monolayer but recognizable intracellular structures have been largely extracted (Fig. 5 *B*). When the temperature of the Triton treatment was raised to 14°C to maximize the extraction of connexin43-NP, morphologically intact gap junctional plaques were still detectable (Fig. 5 *C*), but some appeared to be in the process of dissociating into short, double-membrane structures that became cross-linked to the extracted monolayer upon fixation (Fig. 5 *D*). Morphologically similar disassembly of gap junctions in the presence of detergents has previously been observed in Mauthner cell synaptic disks (Zampighi and Robertson, 1973) and in lens fiber junctions (Kistler and Bullivant, 1988). Thus, although Triton treatment at 14°C affected the ultrastructure of gap junctions, they were still recovered with the extracted monolayer. Taken together, the results depicted in Fig. 4 and 5 indicate that (Triton-insoluble) connexin43-P_2 is localized primarily in gap junctional plaques. Nonphosphorylated, Triton-soluble connexin43-NP appears to be predominantly intracellular, although diffusely distributed connexin43 would not be detectable by morphological techniques.

Transport of Connexin43-NP to the Plasma Membrane in NRK Cells

Although the results depicted in Fig. 4 demonstrated that connexin43-P_2 accumulated on the cell surface in junctional plaques, the cellular location of conversion of newly synthesized connexin43-NP to the P_2 form was not established. Matlin and Simons (1983) found that incubation of mammalian cells at 20°C reversibly blocks transport of nascent secretory and integral membrane proteins within the *trans*-Golgi region. To examine whether phosphorylation of connexin43 and/or its acquisition of Triton resistance occurs before arrival in this intracellular compartment, confluent monolayers of NRK cells were metabolically labeled with [^{35}S]methionine for 4 h at 20°C. Comparison of [^{35}S]methionine–connexin43 synthesized at 20°C (Fig. 6, lanes *1–3*) with that labeled at 37°C (Fig. 1 *A*, lanes *3–5*) revealed that connexin43 remained in the Triton-soluble connexin43-NP state at the lower temperature. Reversal of the 20°C block by incubation of the cells for an additional 2 h in chase medium at 37°C resulted in efficient conversion of [^{35}S]methionine–connexin43-NP to Triton-insoluble [^{35}S]methionine–connexin43-P_1 and -P_2 (Fig. 6, lanes *4–6*). Thus, both

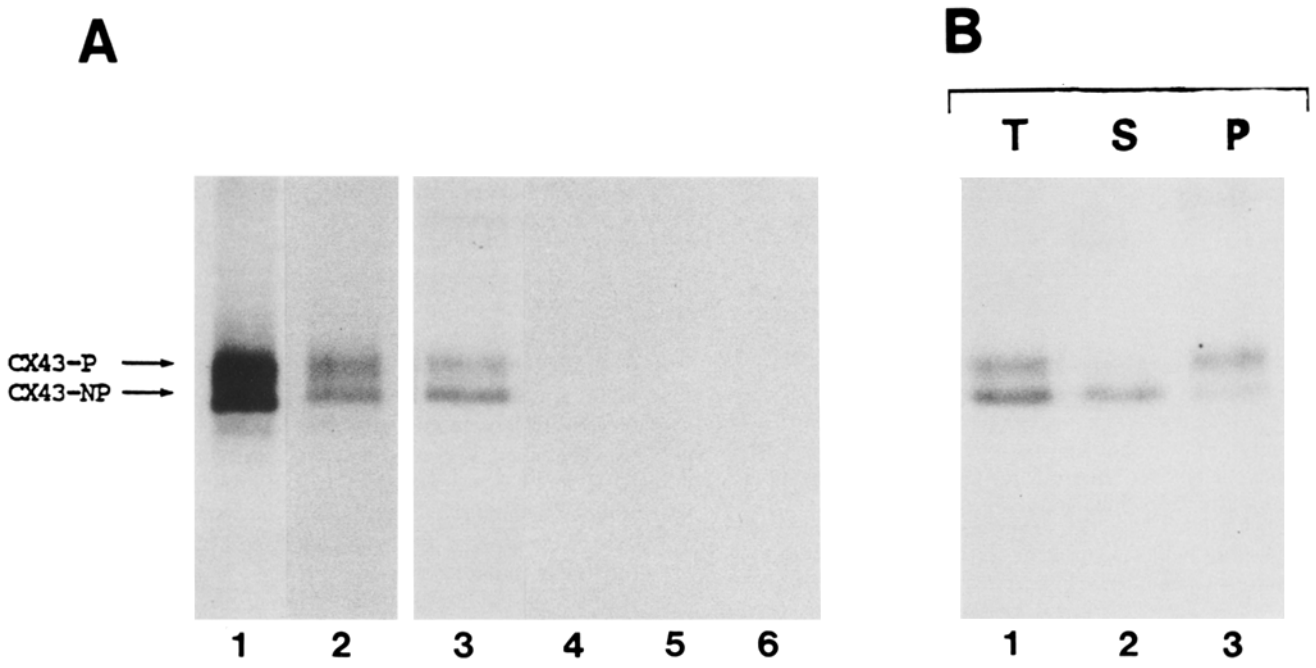


Figure 7. Cell-surface biotinylation of connexin43 in intact NRK cells. Confluent NRK cell monolayers labeled with [^{35}S]methionine at 37°C for 5 h were subjected to cell-surface biotinylation at 4°C. The cells were then either lysed directly into SDS (*A*) or processed for the standard Triton solubility assay (1% Triton in PBS, 30 min, 4°C) (*B*). (*A*) Lane *1*, total cellular connexin43 immunoprecipitated from 1/15 of a biotinylated 60-mm cell culture. Lane *2* is a shorter exposure of lane *1*, illustrating the lack of resolution between the P_1 and P_2 forms of connexin43 after the biotinylation procedure. Lane *3*, biotinylated connexin43 recovered from the remaining 14/15 of the 60-mm cell culture by sequential precipitation with affinity-purified anti-connexin43 (252–271) antibodies followed by avidin–agarose. Same exposure as lane *1*. Lanes *4–6*, same as lane *3*, except connexin43 was immunoprecipitated in the presence of 100 μg/ml of competing connexin43 (252–271) peptide (lane *4*); NHS–LC-biotin was quenched with excess glycine prior to the biotinylation reaction (lane *6*); or NHS–LC-biotin was omitted during the biotinylation procedure (lane *5*). (*B*) Cell-surface biotinylated connexin43 recovered from equal amounts of the total cellular lysate (lane *1*), Triton-soluble supernatant (lane *2*), or Triton-insoluble pellet (lane *3*) fractions.

acquisition of Triton insolubility and phosphorylation of connexin43 to the P_1 and P_2 forms occurred in a cellular compartment distal to the medial Golgi. A similar conclusion was drawn from experiments in which intra-Golgi transport was inhibited with nontoxic concentrations of the carboxylic ionophore monensin (data not shown). In this case, however, the block in connexin43 processing was less complete than at 20°C, presumably because of the known leakiness of monensin's effect on intracellular transport at 0.5–1.0 μM (Peters et al., 1983).

The 20°C block experiments raised the possibility that phosphorylation of connexin43 and/or acquisition of Triton resistance occurred after transport of connexin43 to the cell surface. This possibility was investigated using the technique of cell-surface biotinylation (Le Bivic et al., 1989, 1990*b*) to selectively label and monitor connexin43 on the plasma membrane. [^{35}S]methionine-labeled cell monolayers were incubated at 4°C with the membrane-impermeant protein biotinylating reagent NHS–LC-biotin, which in intact cells reacts covalently with primary amine groups (mostly lysine residues) located in extracellular domains of plasma membrane proteins (Sargiacomo et al., 1989). After 30 min the reaction was quenched with glycine, the cells were lysed in SDS, and connexin43 was immunoprecipitated by our standard protocol. A fraction of the immunoprecipitated connexin43 was reserved as a sample of total cellular connexin43, and the remainder was subjected to a second round of precipitation with avidin–agarose to selectively recover biotinylated connexin43 molecules.

When NRK cells were metabolically labeled with [^{35}S]methionine for 5 h and then biotinylated as described above, two distinct [^{35}S]methionine–connexin43 species were obtained after the double precipitation procedure and analysis on SDS-PAGE (Fig. 7 *A*, lane *3*). The faster-migrating form had the same electrophoretic mobility as total cellular [^{35}S]methionine–connexin43-NP (Fig. 7 *A*, lane *1*) and was soluble in 1% Triton X-100 (Fig. 7 *B*, lane *2*). The other biotin-labeled band comigrated with phosphorylated total connexin43 and was resistant to Triton X-100 (Fig. 7 *B*, lane *3*); this species was converted to connexin43-NP by alkaline phosphatase (data not shown). The biotinylation and precipitation procedures slightly altered the migration of connexin43 on SDS-PAGE such that connexin43-P_1 was not fully resolved from connexin43-P_2 on either 5.5- or 12.5-cm gels. We will therefore refer to phosphorylated forms of biotinylated connexin43 as simply connexin43-P to reflect this ambiguity.

Several control experiments confirmed the specificity of cell-surface biotinylation of connexin43; neither [^{35}S]methionine–connexin43-NP nor [^{35}S]methionine–connexin43-P was recovered if the anti-connexin43 (252–271) immunoprecipitation step was conducted in the presence of an excess of the peptide against which the antibody was raised (Fig. 7 *A*, lane *4*), or if the biotinylation reagent was either omitted (Fig. 7, lane *5*) or quenched with excess glycine before incubation with the cell monolayer (Fig. 7, lane *6*). Similar results were obtained with S180L cells (data not shown), another communication-competent cell type that forms large

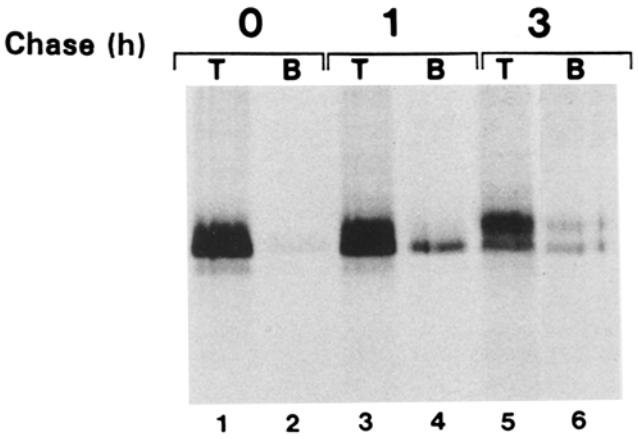


Figure 8. Pulse-chase analysis of transport of connexin43 to the plasma membrane. NRK cell monolayers were metabolically labeled with [^{35}S]methionine for 30 min and chased for 0 (lanes *1* and *2*), 1 (lanes *3* and *4*), or 3 (lanes *5* and *6*) h at 37°C before cell-surface biotinylation at 4°C. Lanes marked *T*, total cellular connexin43 immunoprecipitated from 1/15 of a biotinylated 60-mm cell culture. Lanes marked *B*, biotinylated connexin43 purified by the double precipitation procedure from the remaining 14/15 of the corresponding 60-mm cell culture.

gap junctional plaques (Mege et al., 1988; Musil et al., 1990*b*).

The recovery of cell-surface biotinylated connexin43-NP as well as connexin43-P was consistent with transport of connexin43 to the plasma membrane in the connexin43-NP form. This was confirmed in a pulse-chase experiment in which NRK cells were metabolically labeled with [^{35}S]methionine for 30 min and then chased for 0–3 h at 37°C before biotinylation at 4°C (Fig. 8). Virtually no [^{35}S]methionine-connexin43 became biotinylated immediately after the pulse period (Fig. 8, lane *2*), presumably because most newly synthesized [^{35}S]methionine-connexin43 had not yet reached the plasma membrane and was inaccessible to the biotinylated reagent. This finding indicates that biotinylation was confined to the cell surface, as expected. In contrast, cultures that had been pulsed for 30 min and then chased for 1 h before biotinylation had readily detectable amounts of avidin-precipitable [^{35}S]methionine-connexin43 (Fig. 8, lane *4*). It is significant that nearly all of this biotin-labeled connexin43 was in the connexin43-NP form and was Triton soluble (see Fig. 9 *B*, lanes *1–3*); the minor amount of biotinylated [^{35}S]methionine-connexin43-P recovered at this time was most likely connexin43-P_1 since newly synthesized connexin43-NP required chase periods of >1 h in order for detectable amounts to be converted to the P_2 form (Fig. 3; Musil et al., 1990*b*). When NRK cells were biotinylated after a 3-h chase, [^{35}S]methionine-connexin43-NP and connexin43-P were precipitated in approximately equal amounts (Fig. 8, lane *6*). The decrease in the total amount of biotinylated [^{35}S]methionine-connexin43 recovered at this time relative to that obtained after a 1-h chase was most likely due to a combination of the rapid turnover rate of connexin43 ($t_{1/2}$, ~2–2.5 h) (Musil et al., 1990*b*) and the decreased efficiency with which connexin43 became biotinylated after incorporation into gap junctional plaques (see Discussion). Taken together, these results demonstrated that at least a fraction of connexin43 in NRK cells arrived on the plasma membrane within 1–1.5 h of synthesis and in the Triton-soluble connexin43-NP form.

Phosphorylation of Cell Surface Connexin43-NP in NRK Cells

Cell-surface biotinylation has been shown not to interfere with subsequent intracellular transport and processing of plasma membrane proteins (Matter et al., 1990; Le Bivic et al., 1990*a*, *b*). It was therefore reasonable to study the fate of cell surface connexin43-NP by use of this technique (Fig. 9). NRK cell monolayers were metabolically labeled with [^{35}S]methionine for 30 min and chased for an additional hour at 37°C to allow transport of newly synthesized [^{35}S]-methionine-connexin43 to the plasma membrane. The cultures were then biotinylated at 4°C, after which the reaction was quenched with excess glycine and the cells incubated at 37°C for 0–3 h before cell lysis and purification of biotin-labeled connexin43. Control experiments indicated that NRK cultures remained well coupled for at least 3 h after biotinylation (not shown).

In the absence of a chase period after biotinylation, most (80–90%) of the surface-labeled [^{35}S]methionine-connexin43 was in the connexin43-NP form (Fig. 9 *A*, lane *1*; Fig. 8, lane *4*). When cells were chased for 1 h after biotinylation, the profile of biotin-conjugated [^{35}S]methionine-connexin43 changed both qualitatively and quantitatively (Fig. 9 *A*, lane *2*). The amount of biotinylated [^{35}S]methionine-connexin43 decreased by ~40%, reflecting the rapid turnover rate of total cellular connexin43. Of the surface-labeled [^{35}S]methionine-connexin43 remaining, ~50% was in the connexin43-P form due to a two- to threefold increase in the amount of biotinylated [^{35}S]methionine-connexin43-P between 0–1 h of chase. This phosphorylated connexin43 migrated slightly slower than the minor amount of connexin43-P recovered immediately after biotinylation, a finding consistent with conversion to the P_2 form (compare Fig. 9 *A* lanes *1* and *2*). With increasing chase time after biotinylation (Fig. 9 *A*, lanes *3* and *4*), cell-surface-labeled [^{35}S]methionine-connexin43-NP became undetectable by 3 h (Fig. 9, lane *4*) whereas the levels of biotinylated [^{35}S]methionine-connexin43-P declined more slowly at a rate comparable to that of total cellular [^{35}S]methionine-connexin43-P_2 (Musil et al., 1990*b*). These turnover kinetics suggested that the loss of biotinylated connexin43-NP observed between 1–3 h of chase (lanes *2–4*) was due to phosphorylation to connexin43-P. However, direct degradation of at least a fraction of this biotinylated connexin43-NP without conversion to connexin43-P cannot be definitively ruled out.

Examination of the Triton X-100 resistance of connexin43 revealed that most of the biotinylated [^{35}S]methionine-connexin43 was soluble immediately after biotinylation (Fig. 9 *B*, lanes *1–3*). This observation is consistent with results obtained with total cellular [^{35}S]methionine-connexin43 after a similar pulse-chase protocol (Fig. 3, lanes *4–6*). By 3 h of chase after biotinylation, however, surface-labeled [^{35}S]-methionine-connexin43 was completely insoluble in Triton (Fig. 9 *B*, lanes *4–6*). Taken together, these observations indicate that cell-surface biotinylated connexin43 is converted from Triton-soluble connexin43-NP to a phosphorylated connexin43-P species that was Triton-resistant and therefore likely to be part of a gap junctional plaque. Like total cellular [^{35}S]methionine-connexin43 (Musil et al., 1990*b*), virtu-

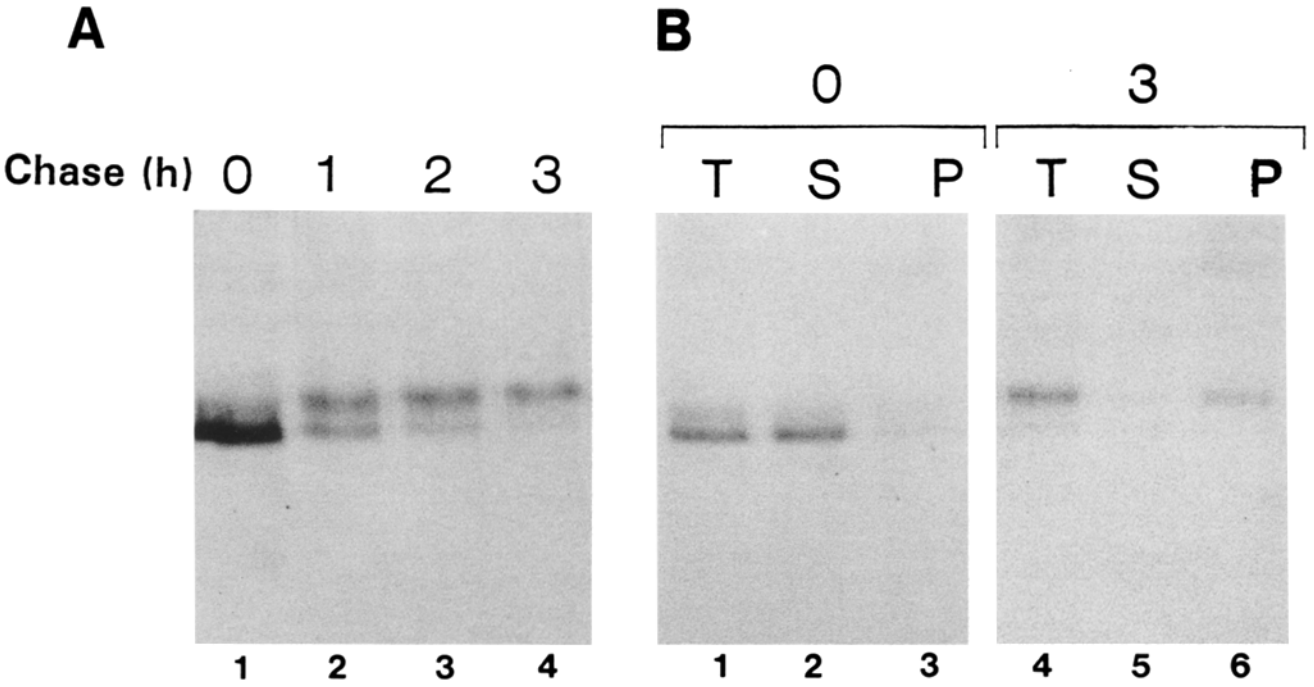


Figure 9. Processing of cell surface connexin43-NP to Triton insoluble connexin43-P. NRK cell cultures were metabolically labeled for 30 min with [^{35}S]methionine and then incubated in the presence of an excess of unlabeled methionine for 1 h at 37°C to permit transport of [^{35}S]methionine–connexin43 to the plasma membrane. The monolayers were then cell-surface biotinylated at 4°C, after which the cells were chased at 37°C for 0–3 h to follow the fate of biotinylated connexin43. (*A*) At the end of the specified chase period, the cells were lysed directly in SDS, and biotinylated connexin43 was recovered by the double precipitation procedure. (*B*) Cells were subjected to the standard Triton solubility assay (1% Triton in PBS, 30 min, 4°C) either immediately after biotinylation (lanes *1–3*) or after a 3-h chase (lanes *4–6*). Cell-surface biotinylated connexin43 was recovered from equal amounts of the total cellular lysate (lanes marked *T*), Triton-soluble supernatant (lanes marked *S*), or Triton-insoluble pellet (lanes marked *P*) fractions.

ally all of the biotinylated connexin43 remaining 4 h after synthesis had undergone this process. We conclude that cell surface connexin43-NP serves as a precursor to connexin43-P, suggesting that transport of connexin43 to the plasma membrane normally precedes phosphorylation to either the P_1 or P_2 form.

Transport of Connexin43 to Plasma Membrane in Communication-Deficient Cell Lines

To determine whether connexin43 was also transported to the surface of communication-deficient cells, confluent monolayers of S180 or L929 cells were metabolically labeled for 5 h with [^{35}S]methionine and then biotinylated at 4°C exactly as described for NRK cells (Fig. 10). A biotin-conjugated species corresponding to [^{35}S]methionine–connexin43-NP was recovered from both cell types, the amount of which (relative to total cellular [^{35}S]methionine–connexin43) ranged from 0.75 to 1.5 times as much as in NRK cells (Fig. 10 *A*, lanes *2* and *6*). Control experiments revealed that radiolabeled connexin43-NP was not appreciably biotinylated immediately after a 30-min pulse with [^{35}S]methionine but became accessible to the biotinylation reagent after a 1-h chase (Fig. 10 *B*; compare lane *2* with lane *4*; lane *6* with lane *8*). Biotinylation of connexin43 thus appeared to be confined to the plasma membrane, as was determined for NRK cells (Fig. 8). We conclude that S180 and L929 cells transport connexin43-NP to the cell surface but are deficient in the ability to assemble it into gap junctions.

Effect of Junctional Communication Disruption on Triton Solubility of Connexin43

Our studies have demonstrated that communication-competent cell types convert connexin43-NP to the Triton-insoluble, plaque-associated P_2 form whereas communication-deficient cells do not. To investigate the dependence of these posttranslational modifications of connexin43 on ongoing cell–cell communication, NRK cells were rendered communication-deficient by treatment with the uncoupling reagent heptanol (Fig. 11). Heptanol has been shown to reversibly block transfer of dye between NRK cells (as well as other connexin43-containing cell types) within 10 min (Chanson et al., 1989). If NRK cultures were labeled for 4 h with [^{35}S]methionine in the presence of 3.5 mM heptanol, none of the [^{35}S]methionine–connexin43 synthesized during this period was phosphorylated to the P_2 form (Fig. 11 *A*, lane *1*). This finding is consistent with our previous results (Musil et al., 1990*b*). This [^{35}S]methionine–connexin43 was soluble in 1% Triton X-100 when assayed under our standard conditions (30 min, 4°C, in PBS) (Fig. 11 *A*, lanes *1–3*). If, however, the cells were labeled with [^{35}S]methionine for 4 h in the absence of heptanol and only then uncoupled by a 10 min incubation in heptanol-containing chase medium, the [^{35}S]methionine–connexin43-P_2 formed during the pulse period remained Triton insoluble and fully phosphorylated (Fig. 11 *B*, lanes *4–6*), indistinguishable from untreated controls (Fig. 11 *B*, lanes *1–3*). Immunohistochemical studies revealed that the macular anti-connexin43 (252–271) stain-

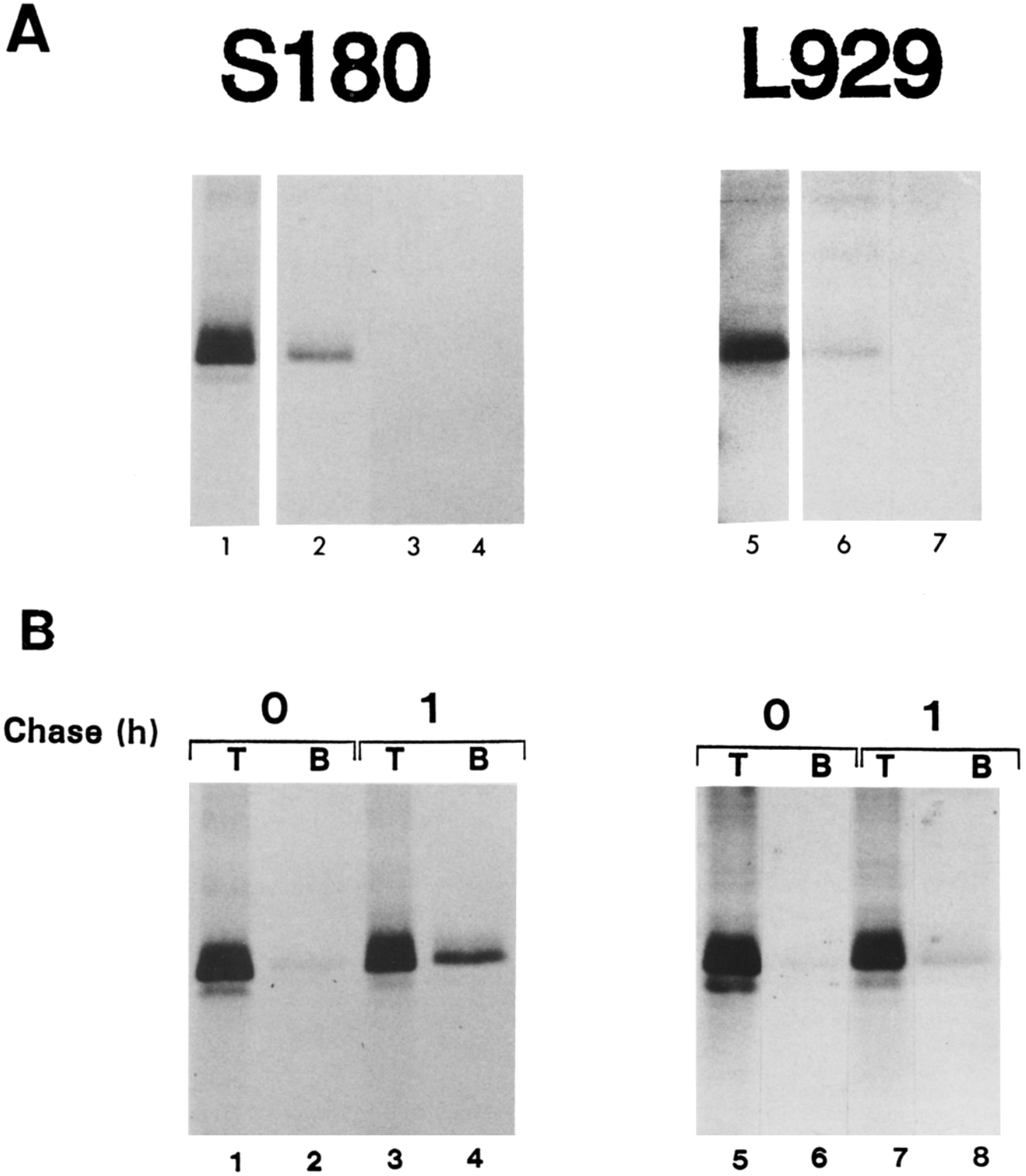


Figure 10. Expression of connexin43 on the plasma membrane of communication-deficient S180 and L929 cells. (*A*) Cultures of S180 (lanes *1–4*) or L929 (lanes *5–7*) cells were metabolically labeled at 37°C with [^{35}S]methionine for 5 h before cell-surface biotinylation at 4°C. The cultures were lysed in SDS and total cellular connexin43 was immunoprecipitated; a fraction of this was then precipitated with avidin–agarose to selectively recover biotinylated connexin43 molecules. Lanes *1* and *5*, total cellular connexin43 immunoprecipitated from 1/15 of a biotinylated 60-mm cell culture. Lanes *2* and *6*, biotinylated connexin43 recovered from the remaining 14/15 of the corresponding 60-mm cell culture. Lanes *3* and *7*, same as lanes *2* and *6*, respectively, except that NHS–LC-biotin was quenched with excess glycine prior to the biotinylation reaction. Lane *4*: same as lane *2*, except that connexin43 immunoprecipitation was conducted in the presence of 100 μg/ml of competing connexin43 (252–271) peptide. (*B*) S180 (lanes *1–4*) or L929 (lanes *5–8*) cells were pulsed for 30 min with [^{35}S]methionine and then chased at 37°C for either 0 h (lanes *1*, *2*, *5*, and *6*) or 1 h (lanes *3*, *4*, *7*, and *8*). Cell-surface biotinylation was then conducted at 4°C. Lanes marked *T*, total cellular connexin43 immunoprecipitated from 1/15 of a biotinylated 60-mm cell culture. Lanes marked *B*, biotinylated connexin43 purified by the double precipitation procedure from the remaining 14/15 of the corresponding 60-mm cell culture.

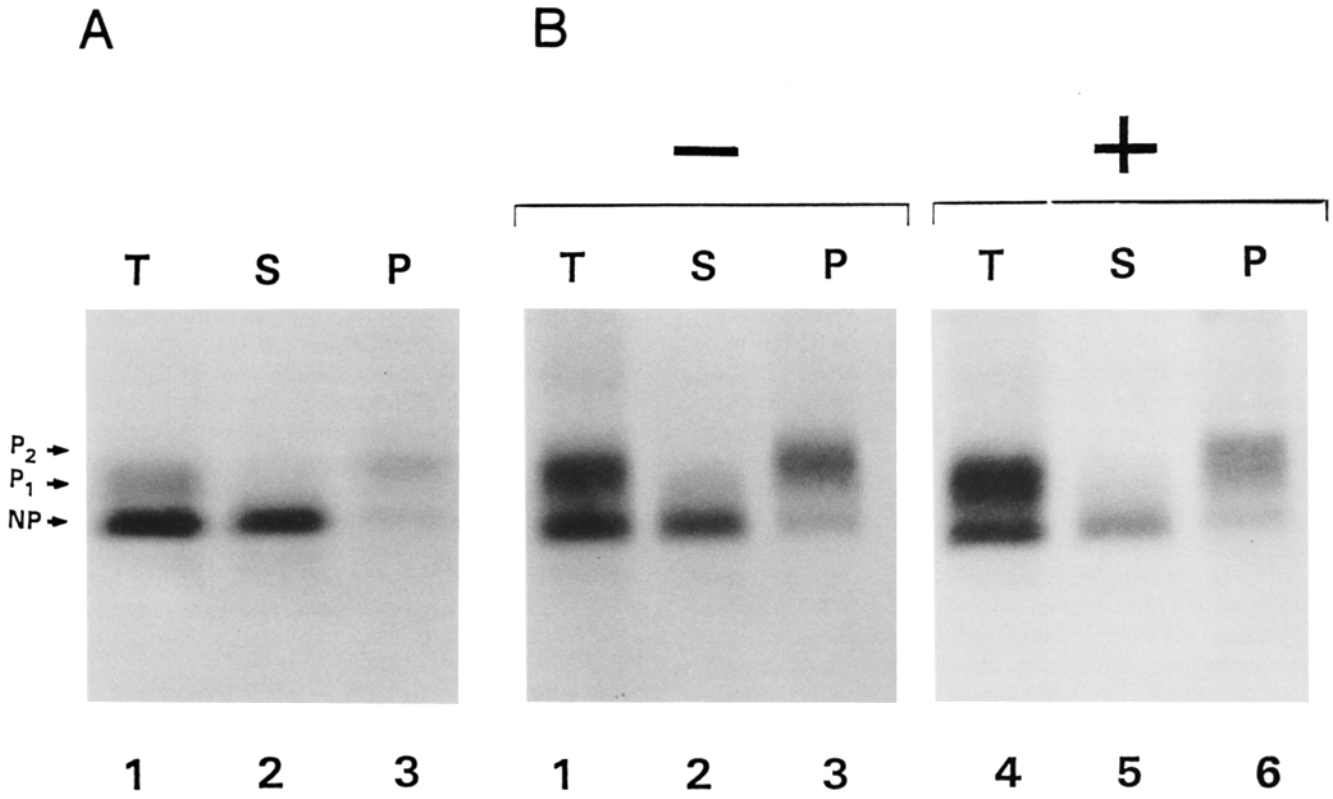


Figure 11. Effect of heptanol on posttranslational processing of connexin43 in NRK cells. Confluent NRK cultures were metabolically labeled for 4 h with [^{35}S]methionine in either the presence (*A*) or absence (*B*) of uncoupling levels (3.5 mM) of heptanol. (*B*) The cultures were then chased for 10 min either without (lanes *1–3*) or with (lanes *4–6*) 3.5 mM heptanol. All cultures were homogenized and subjected to the standard Triton solubility assay (1% Triton in PBS, 30 min, 4°C). Connexin43 was immunoprecipitated from equal amounts of the total cellular lysate (lanes marked *T*), Triton-soluble supernatant (lanes marked *S*), or Triton-insoluble pellet (lanes marked *P*) fractions.

ing pattern observed at cell–cell interfaces in untreated NRK cells was retained after a 10-min incubation in heptanol (data not shown), a finding consistent with the lack of effect of brief exposure to heptanol on the ultrastructure of pancreatic acinar cell–gap junctions (Meda et al., 1986; Bruzzone et al., 1987). Similar results were obtained when NRK cells labeled for 4 h with [^{35}S]methionine were rendered communication-incompetent by a different mechanism, i.e., cytoplasmic acidification induced by brief (10-min) exposure to 100% CO_2 (Schuetze and Goodenough, 1982; data not shown). Thus, under uncoupling conditions, as in all other situations we have tested, connexin43-NP is soluble in Triton in our standard assay whereas connexin43-P_2 is invariably Triton-insoluble.

Discussion

A combination of electron microscopy, x-ray diffraction, and other physical techniques have demonstrated that gap junctional plaques consist of arrays of intercellular channels, each of which is composed of two hexameric connexons joined head-to-head to form a transmembrane pore (Caspar et al., 1988). We have studied the assembly of this complex structure by investigating the intracellular transport and posttranslational processing of connexin43, a member of the closely related family of integral membrane proteins that comprise gap junctions (Beyer et al., 1987; Stevenson and Paul, 1989). We have shown that phosphorylation of connexin43 to the mature P_2 form occurs after arrival of newly synthesized connexin43-NP on the cell surface and is accompanied by accumulation of connexin43 in Triton X-100–insoluble gap junctional plaques. Connexin43 is also transported to the plasma membrane of communication-deficient cells but remains Triton soluble and is not appreciably processed to the connexin43-P_2 form. Taken together, these results are consistent with a model in which connexin43 is constitutively transported to the plasma membrane regardless of the ability of the cell to form gap junctions. Subsequent phosphorylation of connexin43 to the P_2 form occurs only in communication-competent cells and is temporally associated with, and may be functionally involved in, assembly of connexin43 into morphologically and physiologically recognizable gap junctional plaques.

Detergent Solubility of Phosphorylated and Nonphosphorylated Forms of Connexin43

A major finding of this study is that newly synthesized connexin43 is completely soluble in the nonionic detergent Triton X-100 but acquires Triton resistance concomitant with phosphorylation to the connexin43-P_2 form. In situ extraction of communication-competent NRK cultures with 1% Triton, combined with immunofluorescent localization of connexin43, revealed that Triton-insoluble connexin43-P_2 was concentrated in gap junctional plaques whereas nonphosphorylated (Triton-soluble) connexin43-NP was predominantly intracellular. Acquisition of Triton resistance

thus appears to be a useful biochemical marker for accumulation of connexin43 in gap junctional plaques, although we cannot rule out the possibility that a small amount of Triton-insoluble connexin43 (undetectable by immunofluorescence) is present in nonjunctional membranes as well.

The physical basis for the insolubility of connexin43-P_2 in Triton is unknown. Resistance to Triton X-100 is not, however, an inevitable consequence of tight packing of multisubunit integral membrane proteins since the nicotinic acetylcholine receptor remains Triton soluble even after assembly into extremely high-density plasma membrane clusters (Miledi et al., 1971). For some proteins, posttranslational acquisition of Triton insolubility has been shown to reflect association with the cytoskeleton (Nelson, 1989); however, there is no evidence (at least in liver and lens fibers) that gap junctional plaques interact with any filamentous submembranous systems (Hirokawa and Heuser, 1982; Peracchia and Peracchia, 1980). Insolubility of connexin43-P_2 in Triton is also unlikely to be due to formation of intermolecular disulfide bonds since reducing agents fail to convert connexin43-P_2 to a Triton-soluble state (Table I). One potential explanation for the differential solubility of phosphorylated and nonphosphorylated forms of connexin43 is that assembly of connexin43 into gap junctions results in a conformational change in the connexin43 molecule that renders the plaque insoluble in Triton. Alternatively, connexin43 could become posttranslationally associated with Triton-resistant plasma membrane lipids (Yu et al., 1973). Evaluation of this possibility awaits definitive determination of the lipid composition of gap junctions (Malewicz et al., 1990).

The classic method used to isolate gap junctions from rodent liver (composed of connexin32 and connexin26) as well as from heart (containing connexin43) exploits the relative resistance of these structures to solubilization in the ionic detergent *N*-lauryl sarcosine (Goodenough and Stoeckenius, 1972; Hertzberg and Gilula, 1979; Kensler and Goodenough, 1980). The observation here that connexin43-P_2 from NRK cells is virtually quantitatively solubilized under conditions (0.3% sarcosine, 5 mM Tris, pH 10; 10 min, 25°C) that yield sarcosine-insoluble gap junctions from rat heart (Kensler and Goodenough, 1980) thus requires explanation. Differential organization of connexin43 in tissue-culture cells compared to whole organs cannot be the answer, since connexin43 in embryonic chick lens is also soluble in sarcosine in our assay (Fig. 2). Three explanations seem plausible.

First, the gap junctions recovered from rat heart after sarcosine treatment may represent a very small (and perhaps specialized) fraction of the total gap junction population. An early estimate of the yield of gap junctions obtained from mouse liver using a sarcosine insolubility–based isolation procedure was ~10% (Goodenough and Stoeckenius, 1972). The amount of protein recovered in the final gap junction fraction has since been shown to be ~100-fold less (D. Goodenough, unpublished data), decreasing this figure to ~0.1%. Although the reason for this low yield of gap junctions has not been systematically studied, the observation of Hertzberg and Gilula (1979) that rat liver gap junctional plaques are in fact partially soluble in sarcosine suggests that significant loss of junctional material may occur during sarcosine treatment. Consistent with this interpretation, the recovery of gap junctions from rat liver increases 10-fold when a detergent-free, rather than a sarcosine-based, isolation procedure is used (Hertzberg, 1984). Given that cardiac gap junctions are less resistant to sarcosine than those from liver (Kensler and Goodenough, 1980), it is therefore likely that the yield of sarcosine-insoluble gap junctions from heart is very low.

Second, published procedures for the isolation of gap junctions from rodent heart and liver (see above) involve several fractionation steps before sarcosine treatment. It is possible that during these manipulations some gap junctions are artifactually converted to a sarcosine-resistant form in a manner that is not reproduced in our solubilization assay utilizing freshly prepared whole-cell lysates.

A third alternative that we cannot rule out is that connexin43 in heart intercalated disks is genuinely less soluble in sarcosine that connexin43 in either NRK cells or embryonic chick lens epithelium, perhaps reflecting the presence of as yet unidentified connexins or other structural proteins in heart gap junctions that confer stability to harsh detergents. Direct quantitation of the sarcosine solubility of gap junctions in cardiac and hepatic tissue will be required to resolve which of these three possibilities (if any) is correct.

Transport of Connexin43 to the Plasma Membrane

We developed a biochemical assay for transport of connexin43 to the plasma membrane based on the technique of cell-surface biotinylation (Le Bivic et al., 1989 and 1990*b*). Using this assay, we determined that connexin43 is inserted into the plasma membrane of communication-competent NRK cells prior to conversion to either the connexin43-P_1 or the connexin43-P_2 form. This cell-surface connexin43-NP is soluble in Triton, suggesting that transport to the plasma membrane is not coincident with assembly of connexin43 into junctional plaques. This conclusion is further supported by our finding that cell lines with very few (S180 cells) or no (L929 cells) morphologically or physiologically detectable gap junctions nevertheless transport connexin43 to the plasma membrane. Taken together, these studies provide the first biochemical proof for "free" (extrajunctional) channel precursors in the plasma membrane, the existence of which has long been hypothesized (Loewenstein, 1981; Rook et al., 1990). In communication-competent cells, extrajunctional cell-surface connexin43-NP is efficiently converted into Triton-insoluble and therefore plaque-associated connexin43-P_2. Our data thus support a model of gap junction formation in which channel precursors are inserted into nonspecialized regions of the plasma membrane and subsequently accumulate at sites of cell–cell contact by lateral migration in the plane of the membrane bilayer (Loewenstein, 1981). The absence of cell–cell adhesion molecules in communication-deficient S180 and L929 cells (Mege et al., 1988; Nagafuchi et al., 1987) rules out an obligatory role for CAM–mediated intercellular association in the transport of connexin43 to the plasma membrane.

Although highly selective for connexin43 on the plasma membrane, cell-surface biotinylation is not a quantitative technique. In the four cell lines we examined (NRK, S180L, S180, and L929), only ~1% of the total [^{35}S]methionine–connexin43 labeled during a 5-h pulse with [^{35}S]methionine was recovered with avidin–agarose after cell-surface biotinylation at 4°C (Figs. 7 and 10). This low percentage is highly reproducible (range, 0.7–1.3%; $n = 8$ in NRK cells) and is not due to inefficiency of either of the precipitation

steps, incomplete elution of connexin43 from the avidin–agarose beads, or to obvious degradation of connexin43 (data not shown). Two interrelated factors probably account for the limited yield of biotinylated connexin43. First is the low efficiency of the biotinylation reaction itself, which has been estimated to vary from ~9–50%, depending on the particular protein examined (Le Bivic et al., 1989; Matter et al., 1990). In support of this possibility, we have found that raising the temperature at which NRK cells were biotinylated from 4°C to 37°C increased the amounts (relative to total cellular [^{35}S]methionine–connexin43) of avidin-precipitable [^{35}S]methionine–connexin43-NP threefold and of [^{35}S]methionine–connexin43-P tenfold, despite the fact that intracellular connexin43 remained inaccessible to the biotinylation reagent at the higher temperature (not shown). Thus, an absolute maximum of one third of the [^{35}S]methionine–connexin43-NP and one tenth of the [^{35}S]methionine–connexin43-P actually present on the plasma membrane becomes biotinylated under standard labeling conditions (4°C). Should the efficiency of biotinylation of connexin43 at 37°C be considerably less than 100%, this fraction would drop accordingly.

The second probable cause for the low yield of biotinylated connexin43 in communication-competent cells is inaccessibility of plaque-associated connexin43 to the surface-labeling reagent. Comparison of the phosphorylation state of total [^{35}S]methionine-labeled connexin43 in NRK cells with that of cell-surface biotinylated connexin43 reveals that proportionately less connexin43-P than connexin43-NP becomes biotinylated at any given time. Although readily detectable under steady-state labeling conditions (Fig. 7, lane *1* vs. lane *3*), this phenomenon is most strikingly illustrated in a pulse–chase experiment (Fig. 8) in which phosphorylated connexin43 represents 80% of the total cellular [^{35}S]methionine–connexin43 recovered after a 3-h chase (Fig. 8, lane *5*) but only 45% of the connexin43 biotinylated at this time (Fig. 8, lane *6*). Control experiments indicated that this under-representation of connexin43-P after biotinylation was not due to artifactual dephosphorylation during the double precipitation procedure (see Materials and Methods). Since phosphorylated forms of connexin43 are Triton insoluble (Fig. 1 *A*), we believe that the most likely reason why connexin43-P is inefficiently labeled by cell-surface biotinylation is that it is largely incorporated into gap junctional plaques. Plaque-associated connexin43 may be a poor substrate for the biotinylation reagent because of restricted diffusion of NHS–LC-biotin (mol mass = 556 D; 2.24 nm) in the 2-nm intrajunctional "gap" or to changes in the tertiary or quaternary structure of connexin43 that render the protein's reactive lysine residues inaccessible after plaque formation. Reduced biotinylation of junctional connexin43 would also explain why approximately the same fraction (~1%) of total cellular [^{35}S]methionine–connexin43 becomes biotinylated in NRK cells (with abundant large plaques) as in S180 and L929 cells that lack detectable gap junctions.

In light of these quantitative limitations of the cell-surface biotinylation assay, we cannot calculate the $t_{1/2}$ of transport of connexin43 to the plasma membrane or directly determine the fraction of total connexin43 present on the cell surface at a given time. Our demonstration that phosphorylation of connexin43 to the P_2 form occurs after transport to the plasma membrane and that this species accumulates in cell surface plaques does, however, allow us to conclude that most (if not all) connexin43-P_2 is localized to the plasma membrane. Conversely, most intracellular connexin43 appears to be nonphosphorylated, although the presence of internalized connexin43-P_2 destined for degradation cannot be ruled out.

Role of Connexin43 Phosphorylation in Gap Junction Formation and Function

On the basis of our current and previous data (Musil et al., 1990*a* and *b*), we can eliminate certain potential functions for phosphorylation of connexin43 to the P_1 or P_2 form. First, the presence of connexin43-NP on the plasma membrane of both communication-competent and -deficient cells rules out an obligatory role for processing to either the P_1 or P_2 form in the transport of connexin43 to the plasma membrane. Our demonstration that cell-surface connexin43-NP is converted to Triton-insoluble connexin43-P in NRK cells suggests that transport of connexin43-NP to the plasma membrane is part of the normal pathway leading to gap junction formation in communication-competent cells. Since connexin43-P_1 and -P_2 are the only forms of connexin43 to become appreciably labeled with ^{32}P in the cell types we have examined, these results are consistent with arrival of connexin43 at the cell surface in a nonphosphorylated state. The reported presence of an additional, minor phosphorylated form of connexin43 that appears to be a kinetic intermediate between connexin43-NP and -P_1 in vole fibroblasts (Crow et al., 1990) does, however, raise the possibility that in at least some cell types initial phosphorylation of connexin43 may begin before transport to the plasma membrane and conversion to the P_1 and P_2 forms. Second, phosphorylation does not appreciably affect the metabolic stability of connexin43: the $t_{1/2}$ of degradation of connexin43 is equivalent in cells that process connexin43 to both the P_1 and P_2 forms (NRK cells) and those that do not (L929 cells) (Musil et al., 1990*b*). Lastly, phosphorylation of connexin43 is not exclusively associated with actively communicating cells (Fig. 11). The presence of Triton-resistant connexin43-P_2 in uncoupled as well as actively communicating NRK cells suggests that reversible conversion of connexin43 to and from the Triton-insoluble P_2 form is not the mechanism of intercellular channel opening and closure.

Under all conditions examined, terminal phosphorylation of connexin43 was tightly linked to acquisition of Triton insolubility and therefore to accumulation of connexin43 into gap junctional plaques. Phosphorylation to the connexin43-P_2 form could thus potentially be involved in establishment and/or maintenance of gap junctional plaques. In this case, determination of the role of connexin43 phosphorylation will most likely require detailed analysis of the assembly state of phosphorylation-deficient connexin43 mutants.

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