Dynamics of connexin43 phosphorylation in pp60^{v-src}-transformed cells

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Connexin43 phosphorylation was analysed in non-transformed and pp60^{v-src}-transformed Rat-1 fibroblasts. Connexin43 appeared to be the primary connexin expressed in these cells. Although gap-junctional communication was disrupted in pp60^{v-src}-transformed cells, they contained more connexin43 protein and RNA than their non-transformed counterpart. Connexin43 was phosphorylated within minutes of its synthesis in both cell types and appeared to be degraded while in the phosphorylated state. Phosphopeptide and phosphoamino acid analyses suggested that connexin43 in both cell types contained

at least five fragments with serine phosphorylation. The major difference in connexin43 phosphorylation between the pp60^{v-src}-transformed and non-transformed cells was that, whereas approx. 70 % of the phosphorylated connexin43 in the former contained phosphotyrosine, this phosphoamino acid was not detected in connexin43 isolated from the latter cells. These data support the hypothesis that phosphorylation of connexin43 on tyrosine is critical for the blockade of gap-junctional communication which occurs concomitantly with transformation by the pp60^{v-src} oncogene.

INTRODUCTION

pp60^{c-src} is a protein tyrosine kinase which acts as a dominant oncoprotein [1,2]. It contains a negative regulatory site, Tyr⁵²⁷, which is a target of the c-src kinase [3]. The tyrosine kinase activity of pp60^{c-src} is greatly reduced on phosphorylation of this site [4,5]. Virally encoded oncogenic forms of the protein (pp60^{v-src}) have C-terminal truncations and therefore lack this negative regulatory site [6]. The pp60^{v-src} protein contains an N-terminal myristoylation site which is necessary for the ability of the enzyme to associate with the plasma membrane and retain its transforming potential [6].

As pp60^{v-src} is a tyrosine kinase, the identification of significant phosphotyrosine (P-Tyr)-containing substrates is crucial to understanding its transformation process. Potential targets of pp60^{v-src} activity include proteins involved in signal transduction (e.g. GAP [7] and MAP kinase [8]), cytoskeletal organization (e.g. vinculin [9], paxillin [10] and tensin [11]) and membrane channels, and numerous other peptides [12]. The identification of many substrates has permitted the examination of various components of pp60^{v-src} transformation. The required targeting of pp60^{v-src} to the plasma membrane suggests that phosphorylation targets are also membrane associated [12]. One putative target of pp60^{v-src} is the plasma-membrane gap-junction protein connexin43 (Cx43) [13–16].

Connexins are a family of proteins which form intercellular conduits of 1.5 to 2.0 nm diameter known as gap junctions or ‘connexons’. Connexins contain four transmembrane helices which direct the formation of a protein with cytoplasmic N- and C-terminals, an intracellular ‘loop’ and two extracellular loops. The proteins are named according to the predicted molecular mass of their primary translation products based on their cDNA sequence [17–19].

Molecules that pass through gap junctions seem to be involved in the control of cell growth and differentiation, both of which are altered in response to neoplasia. Transformed C3H10T1/2 cells transfected with Cx43 display a reduced growth rate *in vitro*.

Furthermore, growth of these cells is completely arrested when co-cultured with non-transformed cells [20]. Transfection of C6 glioma cells with Cx43 also results in a decreased growth rate that correlates with the extent of Cx43 expression and communication competence [21–23]. An inverse relationship is observed between gap junctional communication (GJC) and the activity of several oncogenes [24,25]. A decrease in GJC results from transformation by the polyoma middle T antigen [26], v-ras, v-mos [27], pp60^{v-src} [14,15,27], and simian virus 40-transforming genes [28,29].

Other agents that foster cell growth and tumour promotion also have a negative effect on GJC [25,30]. These include epidermal growth factor [31–33], transforming growth factor β [32,33] and tumour promoters exemplified by 12-O-tetradecanoylphorbol 13-acetate (TPA) [34–36]. The fact that GJC is reduced within minutes of exposure to these compounds suggests that it may be a prerequisite to ensuing events.

Phosphorylation of Cx43 has been implicated in the actions of epidermal growth factor [31] and TPA [35,37]. However, pp60^{v-src} has the unique characteristic of inducing phosphorylation of Cx43 on tyrosine [13–16]. The P-Tyr content of Cx43 in pp60^{v-src}-transformed cells correlates well with the activity of the enzyme, its ability to reduce GJC and its transforming potential. Various forms of pp60^{v-src} that are unable to transform cells, for example, those lacking the required myristoylation motif, are also ineffective at inducing Cx43 tyrosine phosphorylation and inhibiting communication [13,15]. The use of temperature-sensitive pp60^{v-src} mutants has revealed that Cx43 is phosphorylated on tyrosine within minutes of a shift to the permissive temperature at which activation of pp60^{v-src} kinase activity occurs [15].

The use of site-directed mutants of Cx43 has implicated Tyr²⁶⁵ as a primary tyrosine-phosphorylation site induced by pp60^{v-src} in *Xenopus* oocytes [16]. The fact that agents and oncogenes that ultimately inhibit GJC (e.g. TPA and pp60^{v-src}) can both increase Cx43 production and phosphorylation [30] underlines the importance of kinase activity in the control of GJC. Indeed some

Abbreviations used: Cx, connexin; GJC, gap-junctional intercellular communication; PKC, protein kinase C; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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phosphorylation events appear to be necessary for, or augment, Cx43-mediated GJC [38].

We describe here investigations of Cx43 expression, phosphorylation and GJC in non-transformed and pp60^{v-src}-transformed Rat-1 fibroblasts. Cx43 was the sole gap-junction protein detected in either cell type. Although the transformed cells expressed more Cx43 than the non-transformed cells, they displayed less GJC. Results from phosphopeptide and phosphoamino acid analysis suggest that this paradox results from pp60^{v-src}-induced Cx43 phosphorylation.

EXPERIMENTAL

Maintenance, metabolic labelling and measurement of GJC of cells

Non-transformed or pp60^{v-src}-transformed Rat-1 fibroblasts [39] were grown in Dulbecco's modified Eagle's medium (Gibco/BRL, Bethesda, MD, U.S.A.) supplemented with 5% fetal bovine serum (Gibco/BRL) at 37 °C in an environment of 5% CO₂ and 100% humidity. These cells were a gift from Dr. T. Parsons (University of Virginia, Charlottesville, VA, U.S.A.). Confluent cells were used for all experiments. These were labelled for 2 h with either 150 µCi/ml [³⁵S]Express (NEX-072; NEN, Boston, MA, U.S.A.) or 300 µCi/ml [³⁵S]methionine (NEG-009; NEN) in methionine-free medium (Gibco/BRL). Radiolabelling with H₃³²PO₄ (NEX053; NEN) at 1.0 mCi/ml was performed in phosphate-deficient medium (Gibco/BRL). For pulse-chase experiments, cells were rinsed and fed with complete medium after labelling and allowed to incubate for the appropriate times. After radiolabelling, cells were washed twice with cold PBS and either frozen at -70 °C or used immediately for immunoprecipitation.

To assay GJC, single cells were injected with 10% Lucifer Yellow in 330 mM LiCl using a pneumatic injector (PLI100; Medical Systems, Greenvale, NY, U.S.A.) and a motorized micromanipulator (Ziess). Micropipettes were pulled with a Flaming-Brown P80/PC micropipette puller (Sutter, San Rafael, CA, U.S.A.). Cells were visualized under a phase-contrast inverted microscope equipped with epifluorescence (Nikon Diaphot). GJC was quantified as the number of cells receiving dye, as determined by their fluorescence under u.v. light within 5 min [14]. The possibility of non-junctional membrane permeability was examined by the use of 10% rhodamine dextran (17.2 kDa) in the microinjection solution; Lucifer Yellow passes through gap junctions whereas rhodamine dextran is excluded [40]. Isolated cells were also used as a control for membrane permeability.

Plasmids and antiserum

Plasmids containing entire coding sequences for rat Cx26, Cx32, Cx37, Cx40 and Cx46 were all gifts from Dr. D. Paul (Harvard Medical School, Boston, MA, U.S.A.). A plasmid containing the entire coding sequence for rat Cx43 was a gift from Dr. E. Beyer (St. Louis University, St. Louis, MO, U.S.A.) [41]. A plasmid containing the entire chicken β-actin-coding sequence [42] was a gift from Dr. W. Eldridge (National Cancer Institute, Bethesda, MD, U.S.A.). Inserts were released using appropriate restriction endonucleases, purified (Gene-Clean, BIO-101) after separation by agarose-gel electrophoresis, and ³²P-labelled by random priming (Prime-a-Gene labelling system; Promega).

Cx43-specific rabbit antiserum against peptides comprising residues 368–382 and 241–255 has been described [14]. Cx43-

specific rabbit antiserum against residues 251–271 [43] was a gift from Dr. D. Paul. Cx43-specific rabbit antiserum against residues 100–122 [17] and 1–17 [44] were gifts from Dr. D. Laird (McGill University, Montreal, Canada) and Dr. B. Yancey (Caltech, Pasadena, CA, U.S.A.) respectively. Rabbit antiserum specific for P-Tyr [45] was a gift from D. McCarley (Syntex, Palo Alto, CA, U.S.A.). Normal rabbit serum was purchased from Cappel Labs. Unless stated otherwise, Cx43 antiserum was directed against residues 368–382.

Immunoprecipitation, gel electrophoresis, CNBr cleavage and phosphoamino acid analysis

For immunoprecipitation, cells were lysed directly on the plate, on ice, with RIPA buffer containing 160 µM NaVO₄, 50 mM NaF and 1 mM phenylmethanesulphonyl fluoride. After a total of 10–20 min, the lysate was clarified and immunoprecipitated with rabbit antiserum [14]. Immunoprecipitated proteins were resolved on 11.25 or 7.5–15% polyacrylamide gels [14]. Gels were then either processed for autoradiography, transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.) for cleavage with CNBr, or transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) for phosphoamino acid analysis. Transfer was performed in buffer consisting of 25 mM Tris/HCl (pH 8.2), 192 mM glycine and 15% (v/v) methanol at 200 V.

CNBr cleavage was performed on protein bands localized by autoradiography. CNBr cuts specifically at the carboxyl side of methionine residues [46]. Excised protein bands were cleaved from nitrocellulose membranes as described [47]. The resulting fragments were then resolved by electrophoresis on 24% acrylamide/0.054% bisacrylamide gels [47]. These samples were either processed for autoradiography or transferred to Immobilon-P membranes for phosphoamino acid analysis.

Phosphoamino acid analysis was performed on protein bands localized by autoradiography on Immobilon-P membranes. Subsequent to excision, the proteins were hydrolysed in 5.7 M HCl as described [48]. The hydrolysates were resolved by two-dimensional electrophoresis and the radiolabelled phosphoamino acids were visualized by autoradiography [49].

Western-blot analysis

Plates of confluent cells were rinsed twice with PBS and lysed in a solution containing 1% Nonidet P40, 50 mM iodoacetamide, 1 mM phenylmethanesulphonyl fluoride, 1 mM EDTA, 1 µM leupeptin, 2 µg/ml aprotinin and 0.7 µg/ml pepstatin in borate buffer, pH 8.0 [50]. The lysate was clarified by centrifugation and assayed for protein concentration. Protein (30 µg per lane) was resolved on 11.25% polyacrylamide gels and transferred to Immobilon-P membranes. Electrotransferred gels were stained with Coomassie Blue to determine the transfer efficiency.

The membrane was washed in PBS, and Western-blot analysis was performed with the Western-Light chemiluminescent detection system according to the directions of the manufacturer (Tropix, Bedford, MA, U.S.A.). Subsequent to analysis, membranes were stained with India ink to ensure that lanes were loaded equally and that transfer was uniform.

Quantification of physical data

Total protein content of clarified lysates used for immunoprecipitation or Western-blot analysis was determined by reaction with fluorescamine in a Perkin-Elmer 650-10S fluorescence spectrophotometer [51]. Radioactivity of specific proteins

was determined by scintillation counting of excised bands which were located by autoradiography. The relative intensities of autoradiographic bands on film were measured by image densitometry (Image; NIH).

Northern-blot analysis

RNA was extracted from approx. 1×10^7 cells by a procedure modified from that previously described [52]. After the dishes had been washed twice with PBS, cells were transferred to 2.2 ml microcentrifuge tubes and vortexed in 2.0 ml of a solution containing 2 M guanidinium thiocyanate, 12.5 mM sodium citrate (pH 7.0), 0.25 % Sarcosyl, 0.05 mM 2-mercaptoethanol, 0.05 M sodium acetate (pH 4.0) and 50 % (v/v) phenol (prepared by the method of Maniatis et al. [53]). The solution was then mixed with 0.2 ml of chloroform, set on ice for 5 min and centrifuged at 12000 *g* for 15 min. The aqueous phase was then transferred to a new tube, mixed with 1 vol. of propan-2-ol, incubated at 4 °C for 15 min, and then centrifuged at 12000 *g* for 15 min. The RNA pellet was washed with 75 % ethanol, dried and resuspended in 1 mM EDTA.

RNA (15 µg per lane) was electrophoresed in 1 % formaldehyde-agarose gels and transferred overnight on to a Nytran membrane by capillary action with QuickDraw blotting paper (Sigma, St. Louis, MO, U.S.A.) in 1.5 M NaCl and 0.15 M sodium citrate (pH 7.0) [53]. The post-transfer gel was stained with ethidium bromide [53] to ensure complete transfer. Pre-hybridization (1 h) and hybridization (overnight) was carried out at 42 °C in a solution containing 50 % (v/v) formamide, 0.1 % Ficoll (400 kDa), 0.1 % poly(vinylpyrrolidone) (40 kDa), 0.1 % BSA (fraction V), 0.9 M NaCl, 50 mM sodium phosphate (pH 7.7), 5 mM EDTA, 0.1 % SDS and 150 µg/ml sheared denatured salmon sperm DNA. Duplicate blots were hybridized with 4×10^6 c.p.m. of each connexin-specific probe/ml. After hybridization, blots were washed twice for 15 min in $6 \times$ SSPE ($1 \times$ SSPE = 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, and 1 mM EDTA)/0.5 % SDS at room temperature, twice for 15 min in $1 \times$ SSPE/0.5 % SDS at 37 °C and once for 1 h in $0.1 \times$ SSPE/0.1 % SDS at 65 °C. After exposure, the membrane was stripped in boiling water and re-exposed to ensure probe removal. The same blots were then rehybridized with a probe (1×10^6 c.p.m./ml) containing the entire β -actin-coding sequence [42] as above.

Each probe was analysed for its ability to hybridize specifically to appropriate restriction fragments of its parental plasmid DNA. These hybridizations were conducted simultaneously (in the same bag) with the Northern-blot analysis to ensure the integrity of each probe. The membranes were finally stained with Methylene Blue [53] in order to further demonstrate complete transfer and equal loading.

RESULTS

GJC is decreased in pp60^{v-src}-transformed Rat-1 fibroblasts and is mediated by Cx43

pp60^{v-src}-transformed cells displayed reduced communication levels, as assayed by dye transfer, compared with their non-transformed counterparts. Rat-1 fibroblasts transferred dye to an average of 49 ± 5.4 ($n = 46$) neighbouring cells compared with an average of 13 ± 2.1 ($n = 35$) cells for pp60^{v-src}-transformed cells. Therefore an almost 4-fold reduction in communication was seen concomitant with pp60^{v-src} transformation.

Northern-blot analysis of RNA purified from non-transformed and pp60^{v-src}-transformed Rat-1 fibroblasts revealed that Cx43

was likely to be the primary connexin expressed in either cell type, as Cx26, Cx32, Cx37, Cx40 and Cx46 mRNA were not detected (Figure 1). Quantification of these results indicated that the transformed cells contained a 2.5-fold higher proportion of Cx40 mRNA than the non-transformed cells. This was likely to contribute to the higher Cx43 protein levels observed in the transformed cells (see below).

pp60^{v-src} transformation increases Cx43 levels

Western-blot analysis was performed in order to establish the relative Cx43 protein content in the two cell types. As shown in Figure 2(a), the Cx43 protein resolved as several distinct electrophoretic bands. These included the primary translation product at 43 kDa and its phosphorylated species which migrated at higher apparent molecular masses (discussed below). Quantification of Western-blot analysis indicated that pp60^{v-src}-transformed cells contained 1.9 ± 0.32 ($n = 3$) times as much Cx43 per µg of total cell protein as non-transformed cells (Figure 2a) which may have been due to an increase in Cx43 transcription and/or mRNA half-life (see above). The amount of total protein per cell was also quantified in both cell types which revealed that transformed cells contained 1.7-fold the amount of protein (0.285 ± 0.055 ng per cell, $n = 2$) as the non-transformed cells (0.170 ± 0.10 ng per cell, $n = 2$). These data, when combined with those obtained from Western-blot analysis (described above), indicated that the pp60^{v-src}-transformed cells contained approxi-

Figure 1 Detection of connexin RNA in non-transformed and pp60^{v-src}-transformed Rat-1 fibroblasts by Northern-blot analysis

(a) Total RNA isolated from non-transformed (R) or pp60^{v-src}-transformed (V) Rat-1 fibroblasts was analysed for Cx26, Cx32, Cx37, Cx40, Cx43 or Cx46. The connexin probe used for hybridization for each section of the membrane is indicated at the top. The positions of 18S and 28S rRNA are indicated on the left. (b) The same blots were stripped and reprobed with a cDNA fragment encoding β -actin. The arrow at the right indicates the position of the β -actin RNA.

Figure 2 Analysis of Cx43 in non-transformed and pp60^{src}-transformed Rat-1 fibroblasts

(a) Western-blot analysis of Cx43 protein levels in Rat-1 and pp60^{src}-transformed Rat-1 fibroblasts: 20 µg of total protein from non-transformed (lane 1) or pp60^{src}-transformed (lane 2) Rat-1 cells was allowed to react with Cx43 CT368 peptide antisera. Non-immune serum, which failed to react with Cx43 protein, was used on duplicate blots as negative controls for the experiments (results not shown). Cx43-specific bands are indicated by the brackets. (b) Immunoprecipitation of Cx43 from normal and pp60^{src}-transformed Rat-1 fibroblasts. Each half of the lysate from a plate of non-transformed (lanes 1, 2, 7, 8) or pp60^{src}-transformed (lanes 3, 4, 5, 6) Rat-1 cells, metabolically labelled with either [³⁵S]methionine (lanes 1–4) or ³²P_i (lanes 5–8), was immunoprecipitated with either Cx43 CT368 peptide antiserum (lanes 2, 3, 6, 7) or non-immune rabbit serum (lanes 1, 4, 5, 8). The immunoprecipitated proteins were then resolved by gel electrophoresis. Migration of molecular-mass markers is indicated (kDa) on the left. Apparent molecular masses of the major phosphorylated forms of Cx43 are given on the right. The location of the non-phosphorylated Cx43 primary translation product is indicated by an arrow on the left.

mately 3.4 times as many Cx43 molecules per cell as their non-transformed precursors.

Transformation by pp60^{src} induces differential phosphorylation of Cx43

Cx43 was immunoprecipitated from metabolically labelled cells. As seen in Figure 2(b), radiolabelled Cx43 from both non-transformed and pp60^{src}-transformed Rat-1 cells was modified into species which could be separated by SDS/PAGE. The non-phosphorylated primary translation product from both cell types labelled with [³⁵S]methionine could be seen as a 43 kDa band (Figure 2b, lanes 2 and 3, arrow). However, both cell types also contained species of Cx43 which migrated as bands located at 44, 45, 47 and 50 kDa. Previous investigations indicated that this differential migration was due to protein phosphorylation [14]. Non-specific bands that co-migrated with the 45 and 47 kDa Cx43 phosphoforms were occasionally detected, as demonstrated by their immunoprecipitation with normal rabbit serum from ³⁵S- and ³²P-labelled cells respectively (discussed below).

The major phosphorylated Cx43 species in non-transformed cells migrated as a 45 kDa band (Figure 2b, lane 7), whereas the majority of the phosphorylated species from the pp60^{src}-transformed cells migrated at higher apparent molecular masses. The presence of substantial levels of a 50 kDa form of the protein in the transformed cells was the most noticeable difference (Figure 2b, lane 6). Possibly every molecule of this particular

Figure 3 Phosphoamino acid analysis of phosphorylated Cx43 from (a) non-transformed and (b) pp60^{src}-transformed Rat-1 fibroblasts

Metabolically ³²P-labelled Cx43 was immunoprecipitated and separated by SDS/PAGE. Two-dimensional phosphoamino acid analysis was performed on each band after transfer to Immobilon membranes as described in the Experimental section. Positions of P-Ser (S), P-Thr (T), and P-Tyr (Y) are given. Electrophoresis in the first dimension at pH 1.9 is to the left, and the second dimension at pH 3.5 is towards the top.

phosphoform in the transformed cells contained at least one P-Tyr residue (see below). Phosphoamino acid analysis was performed on each of the Cx43-specific bands. As can be seen in Figure 3, all phosphoforms from both cell types contained phosphoserine (P-Ser). However, each Cx43 phosphoform from the transformed cells contained P-Tyr residues as well. Although the presence of phosphothreonine (P-Thr) was not evident in Figure 3, analysis of ³²P-labelled cleavage products revealed that the 47 kDa and/or 50 kDa species of Cx43 did contain P-Thr (see below).

The majority of phosphorylated Cx43 in pp60^{src}-transformed cells contains P-Tyr

To evaluate the proportion of Cx43 that was phosphorylated on tyrosine in pp60^{src}-transformed cells, successive immunoprecipitations were performed on lysates after metabolic labelling with ³²P_i. For this experiment, equal fractions of lysate underwent three consecutive immunoprecipitations with anti-Cx43, anti-P-

Figure 4 Quantification of proportion of phosphorylated Cx43 that contains P-Tyr in pp60^{src}-transformed Rat-1 fibroblasts

pp60^{src}-transformed cells were labelled with ³²P_i and equal fractions were immunoprecipitated three times consecutively with Cx43 antiserum, P-Tyr antiserum or non-immune rabbit serum (NRS) to clear the lysate of those specific antigens. Each half of the final lysate was then immunoprecipitated with Cx43 (C), P-Tyr (Y)-specific antisera or non-immune rabbit serum (N). 1, 2 and 3 indicate proteins precipitated from the first, second and third immunoprecipitation respectively. Apparent molecular masses of the different Cx43 phosphoforms are indicated on the right.

Tyr or normal rabbit serum in order to clear the lysate of specific antigen. After the third immunoprecipitation, each half of the final lysate was immunoprecipitated with one of the sera not used previously to clear the original lysate.

Comparison of the third immunoprecipitation (Figure 4, lane 3) with the first (Figure 4, lane 1) indicated that antigen specific for each antiserum was effectively removed from the lysate. Lysate that had been cleared of Cx43 still included P-Tyr-containing proteins which migrated between 44 and 50 kDa (Figure 4, lane Y from Cx43-cleared lysate) suggesting that non-Cx43 P-Tyr-containing proteins may co-migrate with Cx43. Therefore the most accurate determination of the fraction of phosphorylated Cx43 that did not contain P-Tyr can be seen by comparing levels of phosphorylated Cx43 remaining after saturation with normal rabbit serum (Figure 4, lane C from NRS-cleared lysate) with that after saturation with P-Tyr-specific antiserum (Figure 4, lane C from P-Tyr-cleared lysate). Quantification of the data, using image densitometry, from two such experiments indicated that $68.5 \pm 4.5\%$ of phosphorylated Cx43 was effectively removed by immunoprecipitation with the P-Tyr-specific antiserum. Therefore approx. 70% of the molecules of Cx43 that were phosphorylated contained one or more P-Tyr residue(s). Attention should be drawn, however, to the 50 kDa species of Cx43 which was almost completely removed by anti-

P-Tyr serum suggesting that most molecules of this phosphoform contained at least one P-Tyr residue.

Cx43 is phosphorylated within the first 15 min of its translation and is degraded in the phosphorylated state

To investigate the temporal nature of Cx43 phosphorylation, cells were labelled with [³⁵S]methionine for 15 min, and then chased with non-radioactive medium for various times. Cx43 was immunoprecipitated from one-half of the lysate from these cells and analysed by gel electrophoresis. Normal rabbit serum was used on the other half of the lysate as negative controls (results not shown) revealing the presence of non-specific protein which co-migrates with the 45 kDa phosphoform of Cx43. This non-specific band has been described by other investigators [54].

At the end of the 15 min radiolabelling period, the 44 kDa phosphoform of Cx43 was already detectable (Figures 5a and 5b). Previous results indicated that the 44 kDa form and other more slowly migrating peptides represent phosphorylated species of Cx43 [14]. The 45 kDa phosphoform became apparent within 1 h of chase in the non-transformed cells (Figure 5a), whereas the transformed cells displayed a less definitive band at 45 kDa after 15 min (Figure 5b). The appearance of the 45 kDa band is difficult to quantify, however, because of the co-migration of the previously described non-specific protein. The 47 kDa phosphoform became apparent after 3 h in the non-transformed cells (Figure 5a), whereas this band was visible from 15 min in the transformed cells (Figure 5b). By 3 h after labelling, aside from the 45 kDa band which was augmented by non-specific protein, the prominent Cx43 phosphoforms were at 47 kDa in the non-transformed (Figure 5a) and 50 kDa in the transformed (Figure 5b) cells. The transformed cells also contained a large proportion of Cx43 which could not be distinguished between non-phosphorylated protein at 43 kDa and the 44 kDa phosphoform (Figure 5b).

In order to ascertain the final form of Cx43, cells were labelled for 2 h and chased with unlabelled medium. These results indicated that, by 9 h after labelling, the majority of Cx43 was present as more slowly migrating phosphoforms in both the non-transformed and pp60^{src}-transformed cells (Figures 5c and 5d respectively). These phosphoforms seemed to appear at the expense of the primary translation product and less phosphorylated species, suggesting them to be slated for proteolytic degradation. The non-specific protein that co-migrates with the 45 kDa

Figure 5 Pulse-chase analysis of Cx43 from non-transformed and pp60^{src}-transformed Rat-1 fibroblasts

Cells were labelled with [³⁵S]methionine for either 15 min (a and b for non-transformed and transformed cells respectively) or 2 h (c and d for non-transformed and transformed cells respectively) and then chased with non-radioactive medium for the times indicated. Cells were lysed and clarified. Each half of the cell lysate was then immunoprecipitated with either Cx43 antiserum or non-immune rabbit serum (results not shown). Immunoprecipitated proteins were resolved by SDS/PAGE, and visualized by autoradiography. Time of chase (h) is given above each lane. Apparent molecular masses (kDa) of the major Cx43 species are indicated on the right of each panel.

Figure 6 Immunoprecipitation of phosphorylated degradation products of Cx43

A series of antibodies were used to precipitate Cx43 from ^{32}P -labelled non-transformed (a) and pp60^{v-src}-transformed (b) Rat-1 cells. Immunoprecipitated proteins were resolved on a 15% polyacrylamide gel. A phosphorylated peptide of approx. 17 kDa is recognized by the antibodies directed against residues 1–17 or 110–122, but not the others. All of the Cx43-specific antisera are able to precipitate intact Cx43. Lanes 1, 2, 3, 4 and 5 are antisera specific for amino acids 1–17, 100–122, 241–255, 251–271 and 368–382 respectively. Non-immune rabbit serum (lane 6) was used as a negative control. Intact Cx43 and the putative 17 kDa degradation product are indicated on the left. Migration of molecular-mass markers (kDa) is indicated on the right. Cell types used for each group of lanes are indicated at the top.

phosphoform (discussed above) appears to have a relatively long half-life which falsely augments the appearance of that species. The estimated half-life of Cx43, as determined by the rate of decay of its total radioactivity, appeared similar in both cell types.

If Cx43 was degraded in its phosphorylated state, then phosphorylated proteolytic fragments may have been produced. In an effort to detect endogenous phosphorylated Cx43 degradation products, lysates of ^{32}P -labelled cells were immunoprecipitated with a series of antisera specific for five different peptides of Cx43. As shown in Figure 6, a phosphorylated peptide of approx. 17 kDa was recognized by antiserum specific for residues 1–17 (Figure 6, lane 1) and 100–122 (Figure 6, lane 2), but not the others. All of the Cx43-specific antisera were able to precipitate non-degraded Cx43 which was not recognized by normal rabbit serum (Figure 6, lane 6). To demonstrate that this 17 kDa peptide was the same as that recognized by antiserum specific for residues 1–17 and 100–122, each antiserum was used to clear ^{32}P -labelled lysate by three consecutive immunoprecipitations before being immunoprecipitated with the other. These results suggest that the same peptide was recognized by both antisera (results not shown). This hypothetical degradation product would begin at residues 1–17 and end between residues 122 and 255.

Cx43 is phosphorylated on several serine residues in both non-transformed and pp60^{v-src}-transformed Rat-1 fibroblasts

CNBr cleavage and one-dimensional phosphopeptide analysis was performed on ^{32}P -labelled Cx43 to investigate further

Figure 7 Phosphopeptide and phosphoamino acid analysis of CNBr-generated Cx43 fragments

(a) Phosphorylated Cx43 was cleaved with CNBr and the fragments were resolved by gel electrophoresis. Lanes 1, 2 and 3 are from the 44, 45 and 47–50 kDa species of Cx43 respectively. Lane 4 contains a non-specific phosphorylated protein which co-migrated with the 47 kDa phosphoform. The cell types are indicated above each set of lanes. Cx43-specific CNBr fragments are indicated on the left and labelled a–e. Migration of molecular-mass markers (kDa) is indicated on the right. (b) Phosphoamino acid analysis of each CNBr fragment from pp60^{v-src}-transformed cells. The panels are labelled a–e referring to the bands seen in (a). Notation in each panel is as described in the legend to Figure 3.

phosphorylation patterns in the non-transformed and transformed cells. The 44 kDa phosphoform appeared to contain one primary phosphorylated CNBr fragment (Figure 7a, lane 1, band d) whereas the 45 kDa phosphoform contained three (Figure 7a, lane 2, bands b, c and d) and the pooled 47 and 50 kDa phosphoforms contained five (Figure 7a, lane 3, bands a, b, c, d and e). It is interesting that the apparent loss of phosphorylation on fragment d in the 45 kDa and higher phosphoforms was more pronounced in the non-transformed cells. Phosphopeptides migrating between 22 and 30 kDa may have resulted from the cleavage of a non-specific protein which co-migrated with the 47 kDa Cx43 phosphoform, as demonstrated by control digestion of a 47 kDa phosphorylated protein immunoprecipitated with normal rabbit serum (Figure 7a, lane 4).

Phosphoamino acid analysis of the individual CNBr-generated bands of Cx43 from pp60^{v-src}-transformed fibroblasts indicated that, whereas all of the fragments contain P-Ser, only a 4.5 kDa band (band e), which appeared to be derived from the 47 and 50 kDa phosphoforms, contained P-Thr (Figure 7b). We are uncertain of the significance of the P-Thr reaction because of the possibility that it may originate from a non-specific protein which occasionally co-migrates with the 47 kDa Cx43 isoforms. Phosphoamino acid analysis revealed no differences in these fragments between the transformed and non-transformed cell types. The Cx43 peptides from the transformed cells lacked P-Tyr. As CNBr has been shown to attack β -hydroxy groups of phosphorylatable amino acids [46], it is possible that the observed lack of P-Tyr was due to its lability under conditions of this cleavage.

Analysis of the protein sequence predicts the creation of seven fragments from CNBr cleavage. These would have predicted molecular masses of 11.2, 3.0, 2.7, 7.8, 7.3, 4.5 and 6.6 kDa. No undigested Cx43 was detected and all of the fragments are within

this range, which is indicative of complete cleavage. CNBr digestion of [³⁵S]methionine-labelled Cx43 generated no detectable fragments (results not shown) which also suggests that cleavage of the protein was complete, as the sulphur group is released from methionine on CNBr cleavage [46].

DISCUSSION

Transformation of Rat-1 fibroblasts by the pp60^{v-src} oncogene resulted in an almost 4-fold decrease in GJC as measured by dye transfer. Data from Northern-blot analysis suggest that Cx43 was the primary connexin expressed in these cells. Transfection of Rat-1 cells with a Cx43 antisense expression vector resulted in a 25-fold decrease in GJC, which is further evidence that Cx43 is the primary functional gap-junction protein in these cells (G. Goldberg and A. Lau, unpublished work). Therefore pp60^{v-src}-induced inhibition of GJC is probably due to modulation of this protein. Paradoxically, Cx43-specific RNA and protein levels were both elevated by pp60^{v-src} transformation to approximately twice the non-transformed cell levels. Cx43 quantity actually increased over 3-fold on a per cell basis in response to pp60^{v-src} transformation. An increase in immunoreactive gap-junction plaques observed at regions of cell-to-cell contact between pp60^{v-src}-transformed cells by immunofluorescent microscopy using Cx43-specific antiserum suggests that the pp60^{v-src}-induced inhibition of GJC does not involve disruption of connexin membrane trafficking or assembly (W. Kurata and A. Lau, unpublished work).

Phosphorylation of Cx43 may be the mechanism by which communication is reduced in response to pp60^{v-src} activity. Pulse-chase analysis demonstrated a gradual increase in the apparent molecular mass of Cx43 phosphoforms beginning within minutes of its synthesis. Cx43 was eventually phosphorylated into several phosphoforms in both the normal and transformed cells, seemingly, at the expense of the primary translation product. The majority of the protein seems to be degraded while in its most phosphorylated state. These data are in agreement with those obtained with cultured rat cardiac myocytes [54] but differ from those obtained with vole fibroblasts which seem to dephosphorylate Cx43 before its degradation [14]. The explanation for this discrepancy is uncertain, but it may result from differences between the two species or cell types. Our observation of an endogenous phosphorylated 17 kDa fragment, which is reactive with antisera against amino acids 1–15 and 122–255, further supports the notion that phosphorylated Cx43 is degraded in Rat-1 fibroblasts. Cx43 half-life was estimated at approx. 2.5 h in both cell types which is also in agreement with that reported for rat cardiac myocytes [54].

Whereas Cx43 from both cell types contained primarily P-Ser, P-Tyr was specifically detected in Cx43 from the transformed cells. This phenomenon has been reported previously for pp60^{v-src}-transformed vole and Rat-1 fibroblasts [14,15]. Approx. 70% of the phosphorylated Cx43 in the transformed cells contained at least one P-Tyr residue per molecule. These data are in agreement with those of Filson et al. [13], who observed that the majority, if not all, of phosphorylated Cx43 contained P-Tyr in v-src-transformed Balb/c 3T3 fibroblasts. On the basis of a comparison of phosphorylated and non-phosphorylated Cx43 from ³⁵S-labelled pp60^{v-src}-transformed cells, we estimate that up to 50% of the total Cx43 may contain P-Tyr.

Furthermore, pp60^{v-src}-transformed cells appeared to contain substantial levels of a phosphorylated Cx43 species which migrated at 50 kDa. Interestingly, practically all of the molecules of this species seemed to contain at least one P-Tyr residue. This

circumstance may be the result of membrane localization of the higher Cx43 phosphoforms [38,55] thereby being exposed to membrane-bound tyrosine kinase activity. These results contrast with the effects of epidermal growth factor [31] and TPA [35,37] which cause reduction of GJC that is associated with more slowly migrating Cx43 phosphoforms resulting from serine phosphorylation.

Although about 70% of the phosphorylated Cx43 from pp60^{v-src}-transformed cells contained P-Tyr, these cells also contained over three times as many molecules of Cx43 per cell as the non-transformed cells. This indicated that the transformed cells contained at least as much non-tyrosine-phosphorylated Cx43 as the non-transformed cells. However, GJC was still decreased approx. 4-fold. This effect could be explained by the possibility that only one tyrosine-phosphorylated Cx43 molecule in the dodecameric connexon structure may be sufficient to inhibit its function. In addition, we have also observed an increase in Cx43 reactivity in the cytoplasm of pp60^{v-src}-transformed cells, which suggests that not all of the Cx43 ends up in the plasma membrane (W. Kurata and A. Lau, unpublished work).

Furthermore, pp60^{v-src}-induced alterations in serine and/or threonine phosphorylation of Cx43 could also play a role in decreasing GJC. *In vitro* experiments suggest that protein kinase C (PKC) is able to phosphorylate a synthetic peptide sequence of Cx43 at Ser³⁶⁸ and Ser³⁷² [56]. As PKC activation (e.g. by TPA) inhibits Cx43-mediated GJC (discussed above), phosphorylation of these, or other, sites may be responsible for this effect. It has been proposed that pp60^{v-src} may utilize PKC in the transformation process [57]. Cells that are deficient in PKC are unable to be transformed by pp60^{v-src} [58], and agents that inhibit PKC are also able to inhibit pp60^{v-src} transformation without affecting its enzyme activity [57].

Phosphopeptide analysis of CNBr-generated fragments of Cx43 purified by metabolically labelled cells indicated that the protein may be phosphorylated on at least five different serine and one threonine residue(s). Although combinations of different phosphorylation sites may cause the same CNBr fragment to migrate as distinct bands, a minimum of three phosphorylation sites would be required to generate five unique modifications of the same fragment. Unfortunately, the chemical cleavage method used made it difficult to immunoprecipitate or immunoblot the Cx43 fragments, and thus it is not possible to identify the fragments by their apparent molecular masses and antibody reactivity.

The method used for phosphopeptide analysis allowed for a direct comparison of Cx43 serine and threonine phosphorylation between the transformed and non-transformed cell types. Although we did not detect major differences in phosphorylation of these amino acids, this may be due to limitations in the sensitivity of the procedures used. However, tyrosine phosphorylation of Cx43 is clearly a major mechanism by which pp60^{v-src} may reduce GJC. Phosphorylation of Cx43 on Tyr²⁶⁵ appears to be involved in the disruption of GJC by pp60^{v-src} in *Xenopus* oocytes [16]. Although non-phosphorylated Cx43 may be transferred to the plasma membrane, serine phosphorylation appears to be necessary for the formation of functional plaques [38,55]. Phosphorylation of Cx43 on tyrosine does not seem to interfere with membrane trafficking or plaque formation (discussed above). The mechanism by which GJC is disrupted as a result of pp60^{v-src} activity therefore is probably the result of some modification of the conformation of the protein in the connexon structure. Further experiments are currently underway which will attempt to identify Cx43 phosphorylation sites in pp60^{v-src}-transformed cells.

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