Specific Proto-Oncogenic Tyrosine Kinases of src Family Are Enriched in Cell-to-Cell Adherens Junctions Where the Level of Tyrosine Phosphorylation Is Elevated

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Abstract. To approach the transmembrane signaling pathway in the cell-to-cell adherens junctions (AJ), AJ-specific tyrosine phosphorylation was analyzed. When various types of rat adult tissues were pretreated with sodium orthovanadate, a potent inhibitor of tyrosine phosphatase, immunofluorescence microscopy showed that anti-phosphotyrosine polyclonal antibody specifically stained the undercoat of the cell-to-cell AJ. This indicates that the tyrosine kinase activity is elevated at the undercoat of the cell-to-cell AJ of adult tissues. To identify tyrosine kinases responsible for the high level of tyrosine phosphorylation at AJ, we have performed in vitro phosphorylation experiments with cell-to-cell AJ isolated from rat liver (Tsukita, Sh. and Sa. Tsukita. 1989. *J. Cell Biol.* 108:31–41) and immunoblotting analyses with specific antibodies for tyrosine kinases. As a result, three proto-oncogenic tyrosine kinases of src family, c-yes, c-src, and lyn kinases, were identified as major tyrosine kinases in the cell-to-cell AJ of hepatocytes. Furthermore, it was immunofluorescently shown that at least two of these kinases, c-yes and c-src kinases, were enriched at the cell-to-cell AJ of various types of cells including hepatocytes. Based on these findings, it is concluded that, in various types of cells, specific proto-oncogenic tyrosine kinases of src-family (c-yes and c-src) are enriched to work as signal mediators in the cell-to-cell AJ where the level of tyrosine phosphorylation is elevated.

THE adherens junction (AJ)[1] represents a specialized class of cell-to-cell and cell-to-substrate associations (14): the adhesion molecules are cadherins (6, 11, 46) and integrins (21, 38), respectively. This type of junction is characterized by the well-developed undercoat on the cytoplasmic surface of the junctional membrane through which actin filaments are tightly associated. AJ is commonly believed to be directly involved in tissue morphogenesis. This feature is, most likely, related to the tight association between AJ and the force-generating actin filament bundles. In addition to these mechanical properties of AJ, the notion has become widespread that AJ plays crucial roles in the control of cell growth and tissue morphogenesis through transmembrane signaling (6, 11, 19, 26, 38, 50). Recently, the information of the adhesion molecule-based signaling in AJ is gradually increasing; for example, specific types of calcium-dependent protease(4) and protein kinase C (24) were immunofluorescently shown to be highly concentrated at the undercoat of the cell-to-substrate AJ, suggesting that the site-specific proteolysis and/or phosphorylation on serine/threonine residues might be important signals used in AJ.

Evidence has been accumulating that tyrosine phosphorylation is another important signal in AJ (10, 15, 17, 18, 27, 29, 31, 36, 45, 50). The relationship between AJ and tyrosine phosphorylation was first illuminated by Rohrschneider (36) who showed that the undercoat of both cell-to-cell and cell-to-substrate AJ of Rous sarcoma virus–transformed cells contained the src gene product, $pp60^{v\text{-}src}$, which is a tyrosine kinase. Furthermore, in the transformed cells, the oncogenic v-abl tyrosine kinase was also found to be associated with AJ (37). Recent progress in anti-phosphotyrosine antibody technique has made it possible to analyze tyrosine-phosphorylated proteins in situ by immunofluorescence microscopy, immunoelectron microscopy, and immunoblotting. With the use of this technique, it was shown that the phosphotyrosine-modified proteins were concentrated at the cell-to-cell AJ in various tissues during development (45). It was also shown that the level of tyrosine phosphorylation was relatively high at AJ in cultured cells, especially after heat shock (30). Based on these findings, at present it is widely accepted that tyrosine phosphorylation works as an important signal at AJ under the special conditions of transformation, development, cell culture, and heat shock. The question naturally arises whether the tyrosine phosphorylation is also an important signal at AJ in normal adult tissues. The

1. *Abbreviations used in this paper*: AJ, adherens junctions; pAb, polyclonal antibody.

results obtained so far with anti-phosphotyrosine antibody appear to indicate that the level of tyrosine phosphorylation is not elevated at AJ of adult tissues.

Recently, we have developed an isolation procedure for the cell-to-cell adherens junctions from rat "adult" liver (47). The structural integrity of AJ was maintained during our isolation procedure through liver cell, crude membrane, bile canaliculi, and AJ fractions. The final AJ fraction thus provides an advantageous in vitro system for studying the signaling events at the cell-to-cell AJ. Our preliminary experiments with this isolated AJ fraction have revealed that the isolated AJ exhibits a high level of tyrosine kinase activity in vitro, although this AJ was derived from the adult tissue. How can we explain this discrepancy? We thought one of the most possible explanations was that the tyrosine phosphatase activity might be high in adult tissues.

In this paper, we first describe evidence showing that the level of tyrosine phosphorylation is really high at cell-to-cell AJ in rat adult tissues. It was shown that the cell-to-cell AJ in various types of rat adult tissues were specifically stained with anti-phosphotyrosine antibody when the tissues were pretreated with sodium orthovanadate, a potent inhibitor of tyrosine phosphatases (44). Second, by in vitro phosphorylation experiments with isolated AJ and immunoblotting analyses, we have identified c-yes, c-src, and lyn kinases of the src family as major tyrosine kinases of isolated AJ of rat adult liver. Finally, we have immunofluorescently shown the relative enrichment of c-yes and c-src kinases in cell-to-cell AJ in various types of cells. We believe this study will give us a clue to understand the signaling pathway in the cell-to-cell AJ while forming and maintaining the structural integrity of various types of tissues in situ.

Materials and Methods

Antibodies

To prepare anti-phosphotyrosine antibodies, the insoluble material obtained after sonication of *Escherichia coli* expressing the v-abl-encoded transforming protein in its tyrosine phosphorylated form was used to immunize rabbits (31, 52). The immune serum was purified on an immunoaffinity column prepared by linking *O*-phospho-L-tyrosine (Sigma Chemical Co., St. Louis, MO) to CnBr-activated Sepharose (Pharmacia LKB Biotechnology, Sweden). The anti-phosphotyrosine antibodies that specifically bound to the phosphotyrosine groups of the column were eluted with 40 mM phenylphosphate (Calbiochem-Behring Corp., La Jolla, CA).

The polyclonal antibody recognizing all members of the src-family kinases was raised against the conserved sequence of the kinase domain of the c-src kinase. The specific anti-c-yes mAb (43), anti-lyn polyclonal antibody (pAb) (54), anti-c-src mAb, and anti-fyn pAb were prepared against the amino-terminal sequence unique to the respective kinase. The antibodies against the undercoat-constitutive proteins of AJ such as vinculin and tenuin were prepared as described previously (47, 49). Anti-EGF receptor was produced against the EGF receptor of A431 cells. Anti-chick erythrocyte spectrin pAb and anti-Ha-Ras mAb (25) were generously provided by Dr. K. Owaribe and Dr. S. Hirohashi, respectively.

Cell Culture

Rat liver cells were primarily cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FCS, essentially by the method described previously (5). Rat epidermal keratinocytes were obtained from newborn foreskins and initiated into culture as described previously (16, 35). Madin-Darby bovine kidney (MDBK) epithelial cells were cultured in DMEM with 10% FCS.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy for phosphotyrosine in rat adult tissues, PBS (150 mM NaCl, 10 mM phosphate buffer [pH 7.5]) containing 0.5 mM sodium orthovanadate was perfused intravenously through the heart (or through the portal vein for liver), and then rat tissues were frozen using liquid N_2. The frozen sections (5-10 μm thick) were cut in a cryostat, mounted on glass slides, air dried, and fixed in 95% ethanol at −20°C for 30 min and in 100% acetone at room temperature for 1 min. After being rinsed in PBS/0.5 mM sodium orthovanadate/1% BSA for 15 min, the sections were incubated with the first antibodies (anti-phosphotyrosine pAb, anti-phosphotyrosine pAb/anti-tenuin mAb [49], or anti-phosphotyrosine pAb/anti-vinculin mAb [Sigma Chemical Co.]) in PBS/0.5 mM sodium orthovanadate/1% BSA for 1 h. They were then washed three times with PBS/0.5 mM sodium orthovanadate/1% BSA/0.1% Triton X-100, followed by incubation with the second antibodies (FITC-conjugated donkey anti-rabbit IgG or FITC-conjugated anti-rabbit IgG/rhodamine-conjugated goat anti-mouse IgG) in 1% BSA/PBS/sodium orthovanadate/0.1% Triton X-100 for 1 h. After being washed in PBS, they were examined with a fluorescence microscope, an Axiophot photomicroscope. In the localization studies of proteins other than phosphotyrosine, sodium orthovanadate was not contained in all solutions.

For indirect immunofluorescence microscopy of cultured cells, rat liver cells primarily cultured on cover glasses were fixed with methanol at −20°C for 10 min, washed in PBS, and permeated with 0.5% Triton X-100/PBS. Primary rat epidermal keratinocytes and MDBK cells on cover glasses were fixed with 1% formaldehyde/PBS at room temperature for 15 min and permeated with 0.2% Triton X-100/PBS. After being washed three times with PBS, cells were soaked in 1% BSA/PBS for 10 min followed by treatment of the first antibodies in 1% BSA/PBS at room temperature for 1 h. As the first antibody, anti-c-yes, or anti-c-src mAb, or anti-lyn pAb was used in the case of the single staining. For the double staining, they were mixed with anti-vinculin pAb or anti-tenuin mAb to obtain the combination of mAb and pAb. The samples were then washed with 1% BSA/PBS three times, followed by incubation with the second antibodies (FITC-conjugated sheep anti-mouse IgG, or FITC-conjugated donkey anti-rabbit IgG, or FITC-conjugated sheep anti-mouse IgG/rhodamine-conjugated goat anti-rabbit IgG) in 1% BSA/PBS. After being washed with PBS three times, the samples were examined in an Axiophot photomicroscope.

The procedure for the immunofluorescence microscopy of isolated bile canaliculi was described previously (49). In the case of anti-phosphotyrosine pAb staining, all solutions used during bile canaliculi isolation and immunofluorescence microscopy contained 0.5 mM sodium orthovanadate.

In Vitro Phosphorylation Reaction

The AJ fraction was obtained from rat livers through crude membrane and bile canaliculus fractions, as described previously (47). For the in vitro phosphorylation reaction, an amount of protein of each fraction containing 100 μg was incubated with 2 μM ATP and 5-30 μCi of [γ-^{32}P]ATP in 60 μl of 10 mM $MnCl_2$/10 mM Pipes (pH 7.0) at 25°C for 15 min. The samples were pelleted at 10,000 *g* for 15 min and processed for one- or two-dimensional gel electrophoresis or for phosphoamino acid analysis.

One- and Two-dimensional Gel Electrophoresis and Autoradiography

Proteins were separated by the one-dimensional SDS-PAGE (28) or two-dimensional NEPHGE (34) methods. For NEPHGE, slab gel electrophoresis was used. The ^{32}P-labeled proteins were detected by autoradiography of the one- or two-dimensional gels. To screen tyrosine-phosphorylated polypeptides, before autoradiography the one- or two-dimensional gels were incubated in alkali (1 M KOH), which preferentially released phosphoserine and phosphothreonine from the gels (7). Autoradiography was performed with Kodak XAR-5 film and intensifying screens (E. I. du Pont de Nemours & Company, Inc., Wilmington, DE).

Phosphoamino Acid Analysis

After one-dimensional gel electrophoresis, the ^{32}P-labeled bands were excised from the gels. The polypeptides of the bands were released from the gels by digesting the gels with TPCK-trypsin (50 μg/ml in 50 mM NH_4HCO_3) and lyophilized. They were then hydrolyzed in acid (6 N HCl), resulting in the release of [^{32}P]phosphoamino acids. After lyophilization, the phosphoamino acids were dissolved in pH 3.5 buffer containing unlabeled

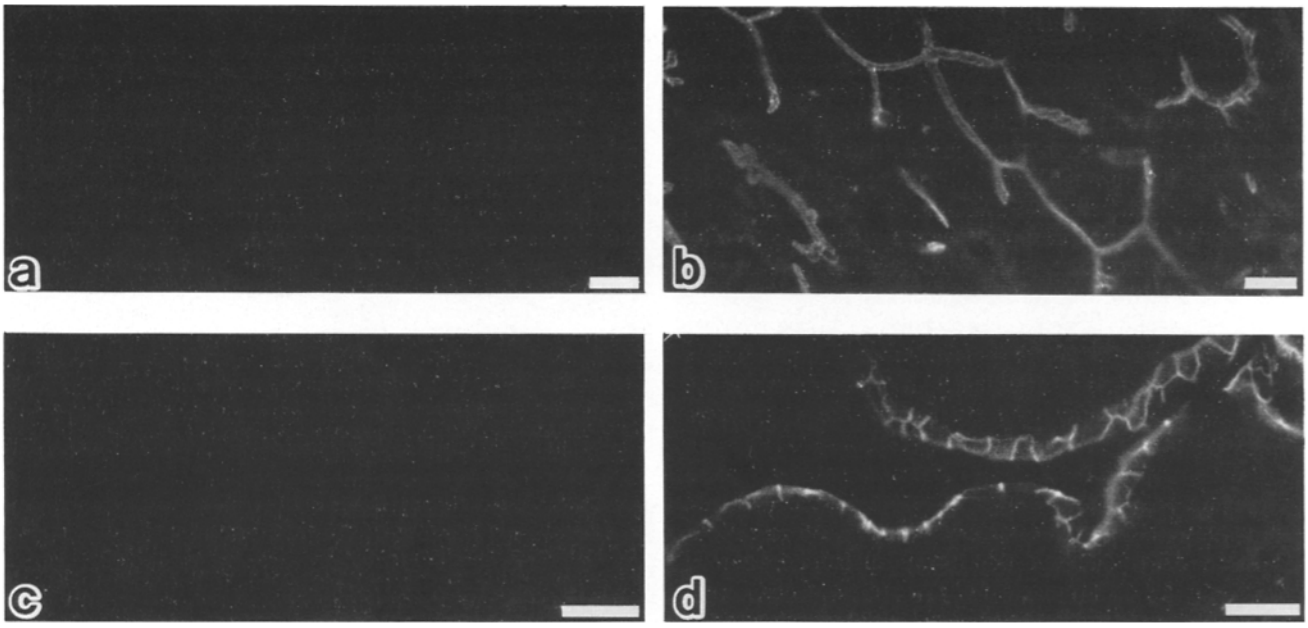

Figure 1. Immunostaining for phosphotyrosine (*a* and *c*) and tenuin (*b* and *d*) on frozen sections of adult rat liver (*a* and *b*) and intestine (*c* and *d*) without pretreatment of sodium orthovanadate. The cell-to-cell adherens junctions are clearly stained by anti-tenuin mAb in both liver and intestine, while no staining was detected with anti-phosphotyrosine pAb. Bars, 10 μm.

phosphoamino acids as internal markers and analyzed by one-dimensional electrophoresis on cellulose thin-layer plates (E. Merck, Darmstadt, Germany) (7). Since we used nuclei-free subcellular membrane fractions such as the adherens junctions, crude membranes, and bile canaliculi, only one-dimensional separation by electrophoresis at pH 3.5 was necessary for identification of [^{32}P]phosphoamino acids.

Immunoblotting

Immunoblotting was performed after one- or two-dimensional gel electrophoresis of isolated AJ and electrophoretic transfer of the polypeptides to nitrocellulose sheets (47, 48). The nitrocellulose sheets were incubated with the first antibody and then treated with second ^{125}I-labeled antibody (Amersham Ltd., Amersham, UK), which was detected by autoradiography.

Results

Concentration of Phosphotyrosine-modulated Proteins at Cell-to-Cell Adherens Junctions in Various Types of Adult Tissues

The level of tyrosine phosphorylation at the cell-to-cell AJ was evaluated by immunofluorescence microscopy with anti-phosphotyrosine pAb. As reported previously (45), when the immunofluorescence microscopy with anti-phosphotyrosine pAb was performed under conventional conditions, no staining was detected at cell-to-cell AJ in various types of adult tissues (Fig. 1). In the present study, to check the possibility that during the procedure for immunofluorescence microscopy most of the phosphates on tyrosine residues are released by tyrosine phosphatases, we have studied the effects of sodium orthovanadate, a potent inhibitor for tyrosine phosphatases (44), on the immunofluorescence staining pattern with anti-phosphotyrosine pAb. For this purpose, before freezing tissues, the physiological saline containing 0.5 mM sodium orthovanadate was perfused intravenously through the heart (or through the portal vein for liver), and 0.5 mM sodium orthovanadate was added to all solutions used for immunofluorescence staining. As a result, in the presence of sodium orthovanadate anti-phosphotyrosine pAb clearly stained the cell-to-cell AJ in various types of adult tissues such as intestinal epithelial cells, liver cells, and cardiac muscle cells, which were identified by anti-vinculin or anti-tenuin staining (Fig. 2). The staining patterns for phosphotyrosine were the same as those obtained by the different kinds of anti-phosphotyrosine antibodies that were kindly provided by Dr. J. Wang (52) and Dr. E. Pasquale (30). The positive labeling for phosphotyrosine was completely inhibited by 10 mM *p*-nitrophenyl phosphate, a ligand of anti-phosphotyrosine antibody (data not shown). It is noted that the phosphotyrosine-staining shows a granular appearance, compared to tenuin- or vinculin-staining. Therefore, to further confirm the idea that anti-phosphotyrosine pAb specifically stained the cell-to-cell AJ, we used the isolated bile canaliculi, which provide an advantageous system for light microscopic identification of the cell-to-cell AJ and desmosomes (49). As a result, when bile canaliculi were isolated and processed for immunofluorescence microscopy in the presence of vanadate, the phosphotyrosine staining, though with granular appearance, coincided with tenuin staining, but not with desmoplakin staining (Fig. 3). Taking all these results together, we were led to conclude that phosphotyrosine was highly enriched in the cell-to-cell AJ of various types of adult rat tissues.

To biochemically evaluate the immunofluorescence results, AJ was isolated from rat adult liver through crude membrane and bile canaliculi fractions, and, after each fraction was incubated in vitro with [γ-^{32}P]ATP in the presence of sodium orthovanadate, the total level of phosphorylation of serine, threonine, and tyrosine residues was analyzed. As is shown in Fig. 4, in both crude membrane and bile canaliculus frac-

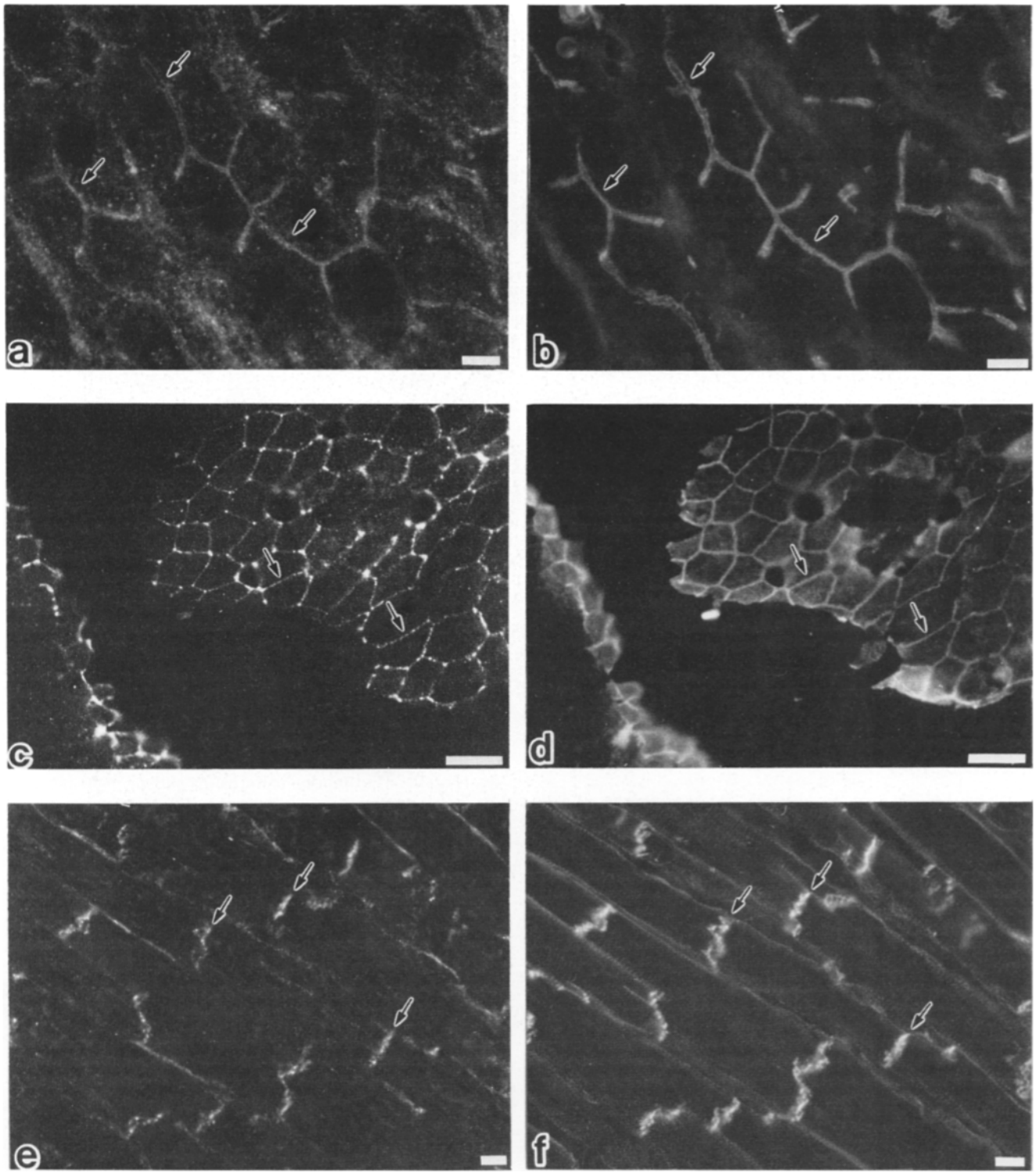

Figure 2. Immunostaining for phosphotyrosine on frozen sections of rat adult liver (*a* and *b*). Intestine (*c* and *d*), and heart (*e* and *f*) after pretreatment with sodium orthovanadate. The frozen sections were stained doubly with anti-phosphotyrosine pAb (*a* and *c*) and anti-tenuin mAb (*b* and *d*) (46) or with anti-phosphotyrosine pAb (*e*) and anti-vinculin mAb (*f*) in the presence of sodium orthovanadate. In these vanadate-treated tissues, the staining pattern with anti-phosphotyrosine pAb is the same as that with anti-tenuin or anti-vinculin mAbs, indicating that the cell-to-cell AJ (*arrows*) is specifically stained with anti-phosphotyrosine antibody. Bars, 10 μm.

tions, serine phosphorylation was dominant, while in the AJ fraction the level of tyrosine phosphorylation was highly elevated.

Taken together, we can conclude that the cell-to-cell AJ of adult tissues are the very regions where tyrosine kinases are highly active. The question has then naturally arisen what kind of tyrosine kinase works as a signal transducer in the cell-to-cell AJ of adult tissues.

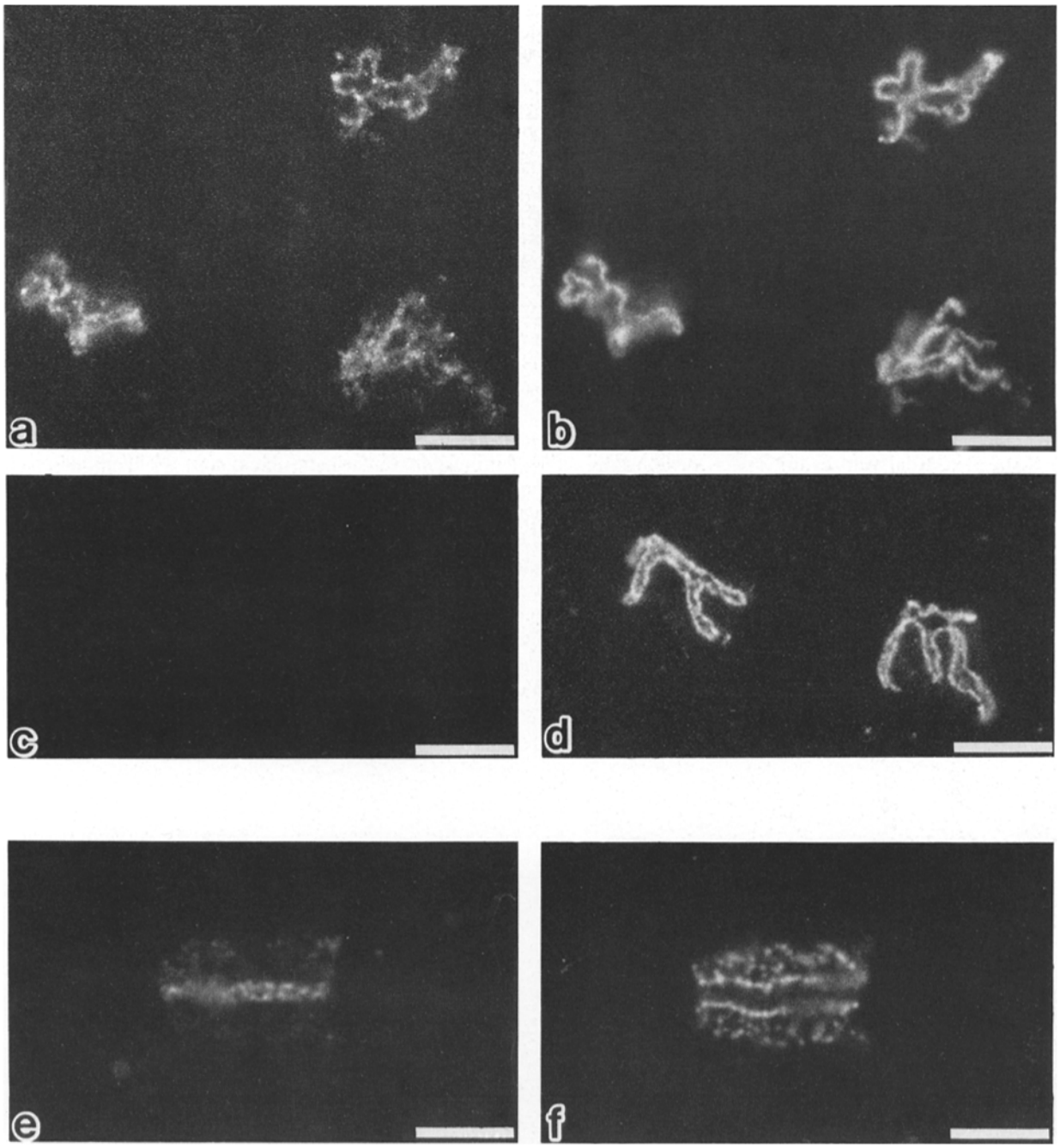

Figure 3. Localization of phosphotyrosine on bile canaliculi isolated from rat liver cells in the presence of vanadate. The isolated bile canaliculi were doubly stained with anti-phosphotyrosine pAb (*a*) and anti-tenuin mAb (*b*), or in the presence of 10 mM *p*-nitrophenyl phosphate (a ligand of anti-phosphotyrosine antibody) with anti-phosphotyrosine pAb (*c*) and anti-tenuin mAb (*d*) or with anti-phosphotyrosine pAb (*e*) and anti-desmoplakin mAb (*f*). These staining patterns reveal that anti-phosphotyrosine pAb exclusively stains the cell-to-cell AJ but not desmosomes, and that the phosphotyrosine staining is completely absorbed by phosphotyrosine analogue (*p*-nitrophenyl phosphate). Bars, 1 μm.

Major Tyrosine Kinases Enriched in Cell-to-Cell Adherens Junctions of Hepatocytes

One of the potent methods for the identification of tyrosine kinases in an isolated fraction is to detect the heavily tyrosine-phosphorylated proteins after in vitro phosphorylation with that fraction, since most tyrosine kinases are known to exhibit a high level of autophosphorylation activity. At present, only from rat liver can the cell-to-cell AJ be isolated. Therefore, we have attempted to identify major tyrosine kinases in this isolated AJ by in vitro phosphorylation experiments.

After in vitro phosphorylation of AJ-fraction using [γ-^{32}P]ATP, the ^{32}P-polypeptides were examined by gel electrophoresis and autoradiography. On the one-dimensional gel, a large percentage of the total radioactivity incorporated was present in five bands with apparent molecular masses between 50 and 62 kD (Fig. 5 *a*, lanes *1* and *2*). Lower levels of ^{32}P labeling occurred in several bands with a wide range of molecular masses in the one-dimensional gel. To charac-

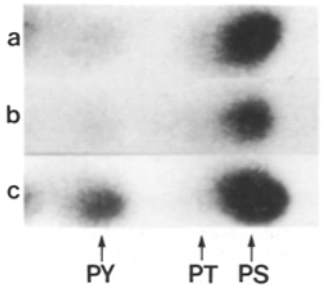

Figure 4. Total ^{32}P-phosphoamino acid analyses of the crude membrane (lane *a*), bile canaliculus (lane *b*), and AJ fractions (lane *c*) which were phosphorylated in vitro. The positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated by *PS*, *PT*, and *PY*, respectively.

terize the tyrosine-phosphorylated polypeptides, the gels were incubated in alkali to preferentially release the ^{32}P of the phosphoserines and phosphothreonines. In the alkali-treated gel, most of the ^{32}P label remained in three bands of 62, 58, and 56 kD, while minor amounts of label were detected in several bands in wide ranges of molecular masses (Fig. 5 *a*, lane *3*). Partial acid hydrolysis of these alkali-resistant ^{32}P-labeled polypeptides released [^{32}P]phosphotyrosine residues which were identified by thin-layer electrophoresis (Fig. 5 *b*). The major [^{32}P]tyrosine-phosphorylated bands of AJ were further analyzed on two-dimensional gels with alkali treatment (Fig. 6). The 62- and 58-kD bands each focused as single spots (designated pp62 and pp58, respectively), while the 56-kD band was resolved into three separate spots of different charges (pp56a, pp56b, and pp56c from the acidic side). The species pp62 and pp58 were similar in charge; between these spots we detected a spot of 60 kD (pp60).

It is widely accepted that normal cells contain two types of membrane-associated tyrosine kinases: receptor type and nonreceptor type, the latter closely related to the src kinase (22, 23). The members of the src family (the c-yes, c-src, fyn, c-fgr, lyn, lck, hck, blk, and tkl kinases) (13, 19, 22, 23, 42, 54) possess molecular masses between 50 and 65 kD and highly homologous kinase domains at their COOH termini. Therefore, using pAb raised against the kinase domain of c-src that can react with all members of src family kinases, we addressed the possibility that the major tyrosine-phosphorylated polypeptides (pp62, pp60, pp58, and pp56a, b, c) identified in Fig. 6 were themselves tyrosine kinases of the src family. This antibody clearly reacted with pp62 and pp58 on the one-dimensional gels by immunoblotting (data not shown), indicating that two different src-like tyrosine kinases (pp62 and pp58) mainly occurred in cell-to-cell AJ. Next, using antibodies specific to each src-like kinase, we identified pp62 and pp58 as the c-yes (42) and lyn (54, 55) kinases, respectively, on two-dimensional gels (Fig. 7 *a*). Furthermore, when a large amount of isolated AJ was loaded on gels and immunoblotted with anti-c-src antibody, a positive band of 60 kD was detected (Fig. 7 *c*), suggesting that pp60 (identified in Fig. 6 *b*) was the c-src kinase. Hence, we concluded that three proto-oncogene products, the c-yes, lyn, and c-src kinases, were associated with cell-to-cell AJ and that the amount of the c-src kinase was smaller than that of the c-yes

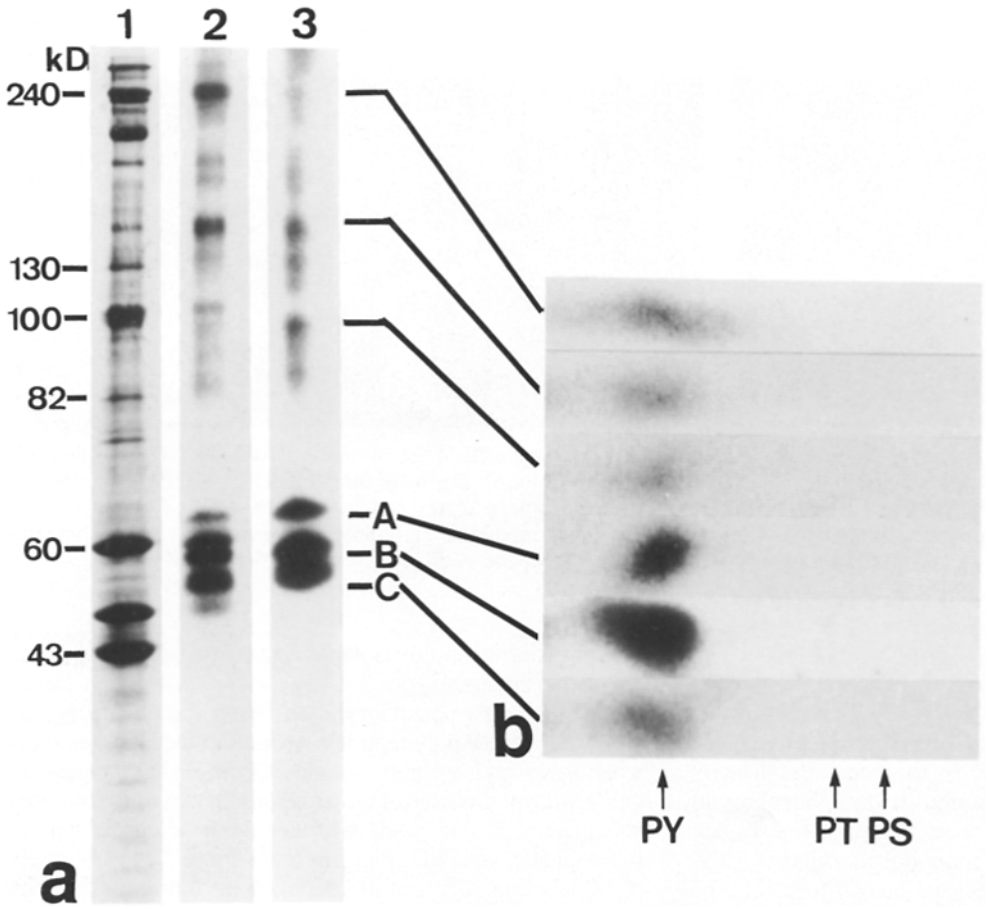

Figure 5. In vitro tyrosine phosphorylated polypeptides in the isolated AJ. (*a*) Coomassie brilliant blue-stained one-dimensional gel of isolated AJ which was phosphorylated in vitro (lane *1*) and corresponding autoradiograms of ^{32}P-labeled polypeptides before (lane *2*) and after (lane *3*) alkali treatment of the gel. In lane *1*, the polypeptides of 130, 100, and 82 kD correspond to vinculin, α-actinin, and radixin, respectively. In lane *3*, note the major tyrosine-phosphorylated bands with apparent molecular masses of 62 (*A*), 58 (*B*), and 56 kD (*C*). Minor tyrosine phosphorylation occurred in the bands around 240, 95, and 160 kD as indicated. (*b*) ^{32}P-phosphoamino acid analyses of the ^{32}P labeled bands of the 240-, 160-, 95-, 62-, (*A*), 58- (*B*), and 56-kD (*C*) proteins that were indicated in lane *3* of *a*. The positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated by *PS*, *PT*, and *PY*, respectively.

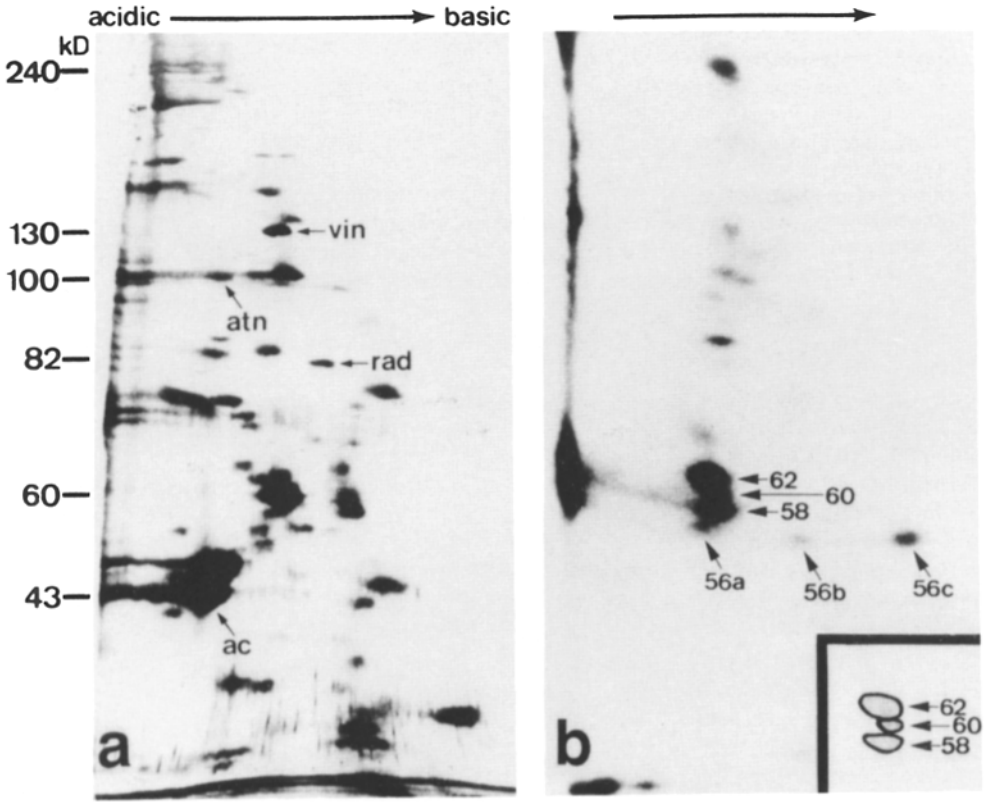

Figure 6. Silver-stained two-dimensional gel (*a*) of isolated AJ that was phosphorylated by [γ-^{32}P]ATP in vitro, and corresponding autoradiogram (*b*) after alkali treatment of the gel. (*a*) The total pattern of polypeptides in isolated AJ. Arrows indicate vinculin (*vin*), α-actinin (*atn*), radixin (*rad*) (48), and actin (*ac*), respectively. (*b*) The pattern of tyrosine-phosphorylated polypeptides on the alkali-treated two-dimensional gel. The major 62- and 58-kD spots (*A* and *B* in lane *3* of Fig. 5 *a*, respectively) focused as single spots (62 and 58, respectively), while the 56-kD band (*C* in lane *3* of Fig. 5 *a*) was resolved into three separate spots of different charges (56a, 56b, and 56c). Between the 62- and 58-kD spots, we detected a weaker spot of 60 kD (60), as shown schematically in the *inset*.

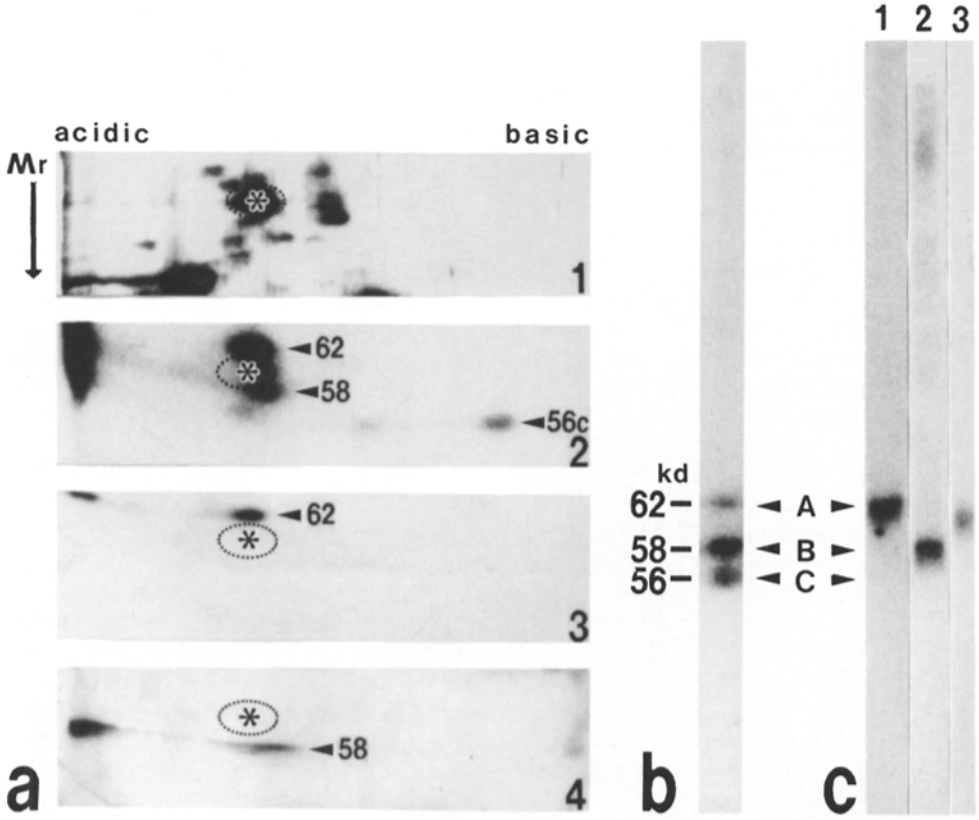

Figure 7. Identification of the major tyrosine-phosphorylated polypeptides of isolated AJ by Western blotting. (*a*) Parts of two-dimensional gels of isolated AJ corresponding to the region of molecular mass 45–65 kD. Section *1*, silver-stained gel of isolated AJ. The major 60-kD spot (*asterisk*, not identical to pp60) was used as a position marker in sections *2–4*. Section *2*, autoradiogram of an alkali-treated gel which shows the major [^{32}P]tyrosine-phosphorylated polypeptides of isolated AJ. *62*, pp62; *58*, pp58; *56c*, pp56c. Sections *3* and *4*, corresponding immunoblots probed with anti-c-yes (section *3*) and anti-lyn (section *4*) antibodies. The positions of positive spots were determined with reference to the 60-kD spot (*asterisks*) which had been identified by staining with Ponceau S before immunoblotting. (*b*) Autoradiogram of a one-dimensional alkali-treated gel showing [^{32}P]tyrosine-phosphorylated polypeptides in isolated AJ (same as lane *3* in Fig. 5 *a*). Note the three major tyrosine-phosphorylated bands (*A*, *B*, and *C*). (*c*) Western blots of isolated AJ probed with anti-c-yes mAb (lane *1*), with anti-lyn pAb (lane *2*), and with anti-c-src mAb (lane *3*).

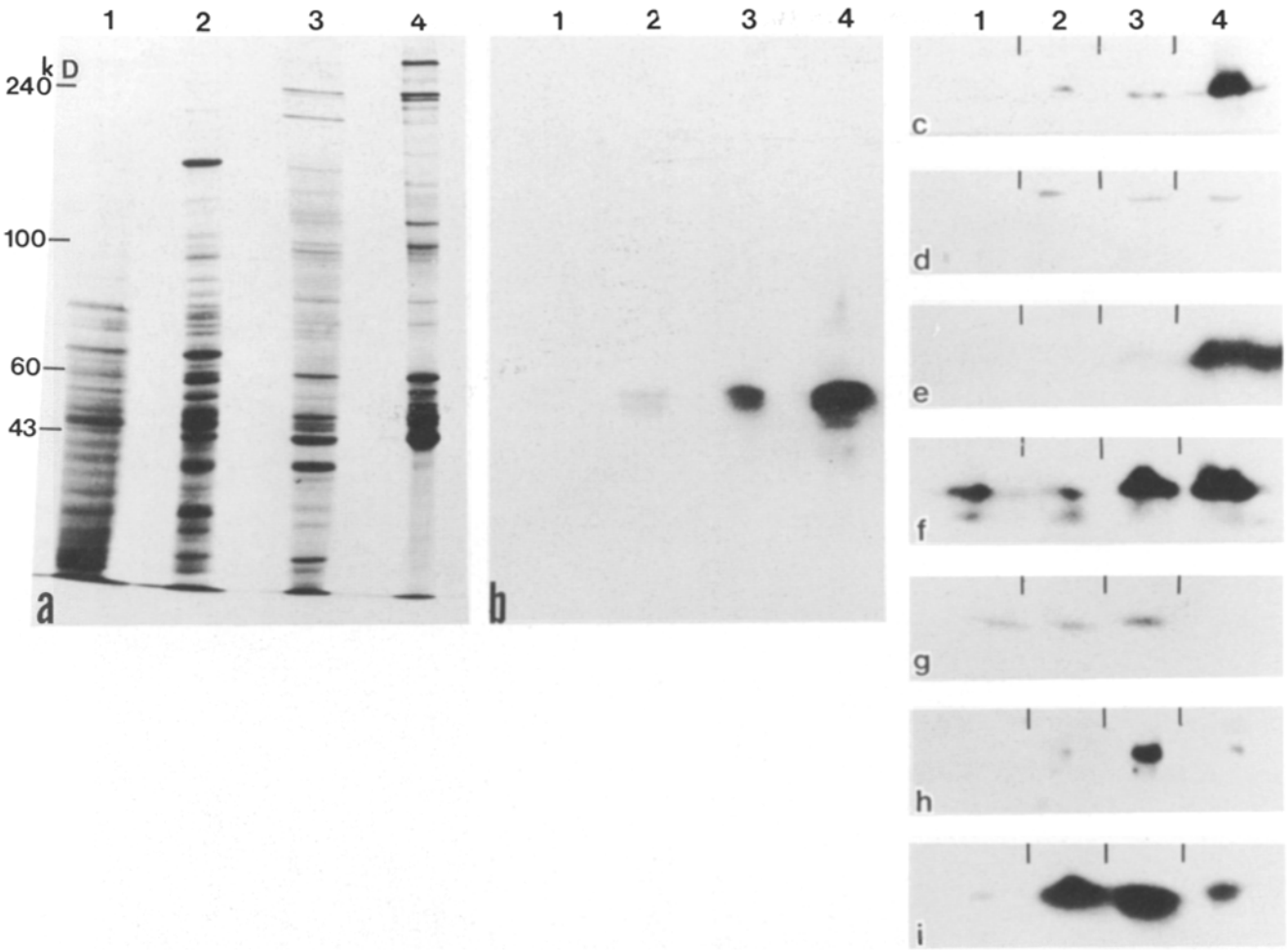

Figure 8. Enrichment of the c-yes, lyn, and c-src kinases in AJ. (*a*) Coomassie brilliant blue–stained gels. Fractions of liver cells (lane *1*), crude membranes (lane *2*), bile canaliculi (lane *3*), and AJ (lane *4*) were loaded on one-dimensional gels with the same amount of total protein. (*b–i*) Corresponding immunoblots with anti-lyn pAb (*b*), anti-vinculin pAb (*c*), anti-spectrin pAb (*d*), anti-c-yes mAb (*e*), anti-c-src mAb (*f*), anti-fyn pAb (*g*), anti-EGF receptor pAb (*h*), and anti-Ha-ras mAb (*i*).

and lyn kinases in isolated liver AJ. Consistent with this result, the use of [α -^{32}P]8-azidoadenosine 5-triphosphate (8-N_3ATP) (12), a photoaffinity analogue of ATP, revealed ATP-binding spots around pp62, pp60, and pp58 on two-dimensional gels (data not shown). The identity of the three 56-kD spots (pp56a, pp56b, and pp56c) remains to be determined; none of them reacted with the anti-kinase domain of src-family kinases or with 8-N_3ATP.

Next, to determine whether these three src family tyrosine kinases (c-yes, c-src and lyn kinases) were exclusively enriched in AJ or present at high levels in the nonjunctional regions of liver cell plasma membranes as well as in AJ, on one-dimensional gels we separated fractions of liver cells, crude membranes, bile canaliculi, and AJ (each containing the same amount of total protein), and immunoblotted them with different antibodies. As shown in Fig. 8, all of these three kinases were highly enriched during the isolation procedure, similar to the case of vinculin. It was of special interest that the other src-family tyrosine kinases expressed in adult liver, such as the fyn kinase, were not enriched in AJ. Furthermore, the EGF receptor kinase and the c-Ha-ras protein (25), like spectrin, were not enriched in the AJ fraction (Fig. 8).

Hence, we are led to conclude that major tyrosine kinases in the cell-to-cell AJ of adult liver are c-yes, c-src, and lyn kinases, and that these kinases are highly concentrated at cell-to-cell AJ. Thus, the next question is whether these src-like kinases occur in the cell-to-cell AJ of various types of cells other than liver cells.

Enrichment of c-yes and c-src Kinases at Cell-to-Cell Adherens Junctions in Various Types of Cells

Since so far the isolation procedure for the cell-to-cell AJ was available only for the adult liver, it is impossible at present to determine biochemically whether c-yes, c-src, and lyn kinases occur in the cell-to-cell AJ of other tissues. Therefore, we have attempted to analyzed immunofluorescently the distribution of these kinases by the use of frozen sections of AJ-bearing tissues. However, as can easily be expected, the signals from specific antibodies were very weak, probably owing to the small amounts of these kinases. For example, anti-c-yes and anti-c-src kinases only faintly stained the cell-to-cell AJ in the frozen sections of liver (data not shown). Thus, we have decided to use cultured cells for immunofluorescence study, since in cultured cells the sensitiv-

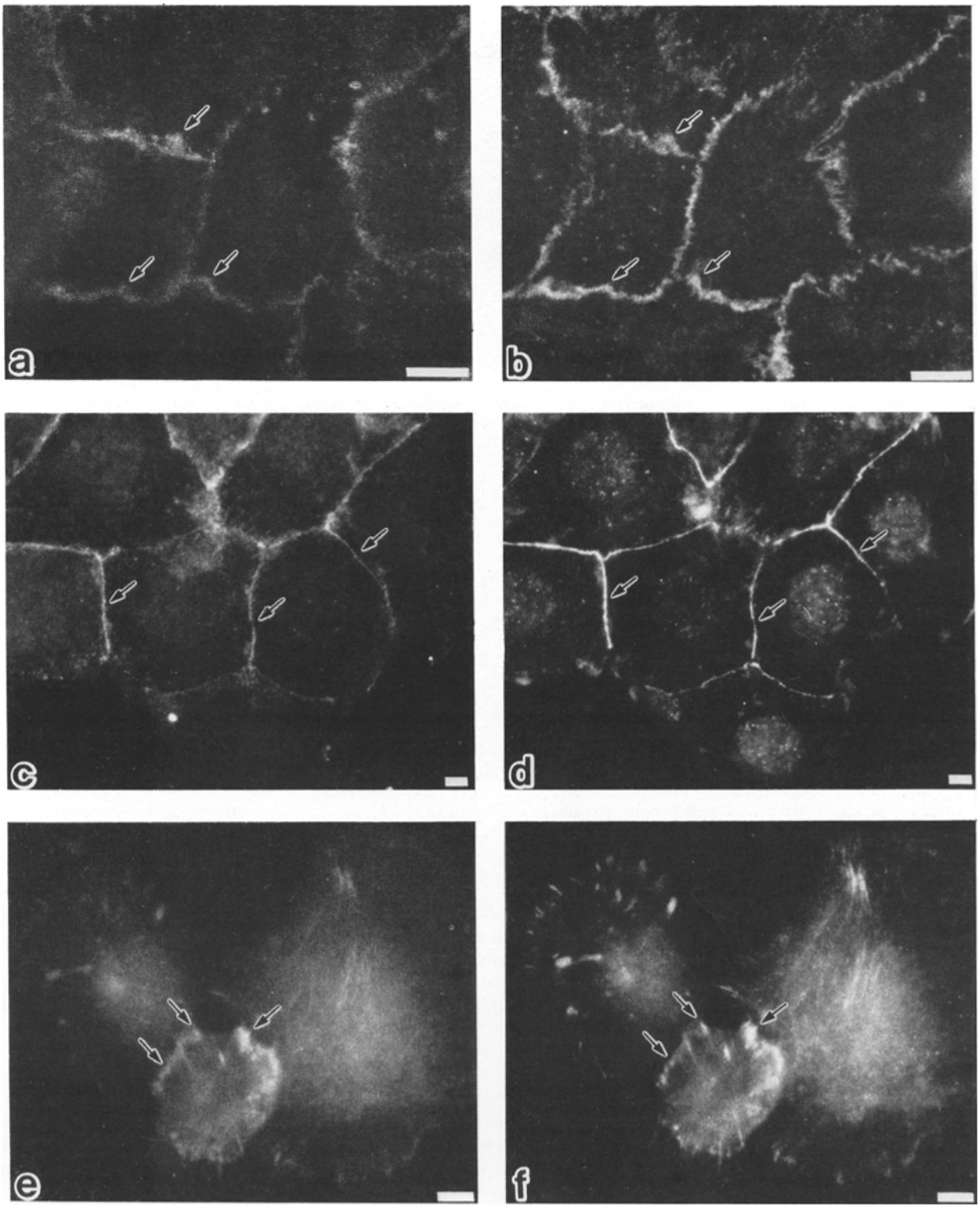

Figure 9. Immunostaining for the c-yes kinase in cultured cells. Primarily cultured rat liver cells (*a* and *b*), MDBK cells (*c* and *d*), and primarily cultured rat keratinocytes (*e* and *f*) were doubly stained with anti-c-yes kinase mAb (*a*, *c*, and *e*) and anti-vinculin pAb (*b*, *d*, and *f*). Note that c-yes is concentrated at the cell-to-cell AJ (*arrows*) that are identified by vinculin staining. Bars, 10 μm.

ity for the detection of staining is clearly higher than in frozen sections, mainly because the cultured cells on the cover glasses are quite thin. For this purpose, we have chosen three types of cell-to-cell AJ-bearing cells: primarily cultured hepatocytes, MDBK cells (epithelial cells from kidney), and primarily cultured keratinocytes. At first, these cultured cells were stained with anti-c-yes kinase mAb in the presence or absence of anti-vinculin pAb (47) (Fig. 9). In all of these cells, the staining signals of anti-c-yes kinase mAb were fully detectable, and they coincided with those of anti-vinculin

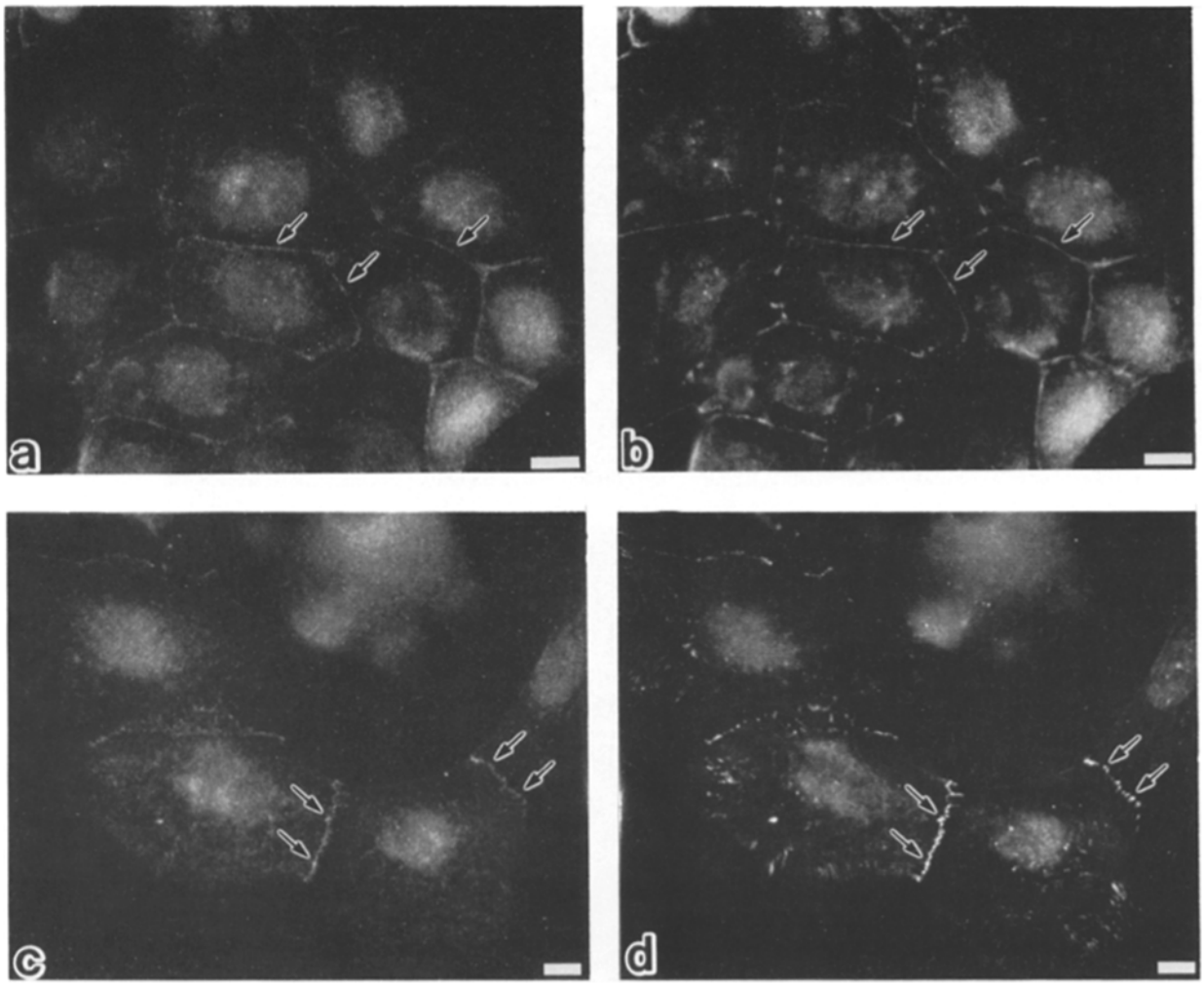

Figure 10. Immunostaining for the c-src kinase in cultured cells. MDBK cells (*a* and *b*), and primarily cultured rat keratinocytes (*c* and *d*) were doubly stained with anti-c-src kinase pAb (*a* and *c*) and anti-vinculin mAb (*b* and *d*). The cell-to-cell AJ (*arrows*) is exclusively labeled by both anti-c-src mAb and anti-vinculin pAb. Bars, 10 μm.

pAb, indicating that the c-yes kinase is concentrated at the cell-to-cell AJ in these cells. Next, these cells were stained with anti-c-src kinase mAb in the presence or absence of anti-vinculin pAb (Fig. 10). The results obtained were the same as those from anti-c-yes kinase mAb, showing that c-src kinase was also enriched in the cell-to-cell AJ in these cells. Finally, we have analyzed the distribution of the lyn kinase in these cells. However, the labeling by anti-lyn kinase pAb was too weak to determine the distribution pattern of the lyn kinase in these cells. Only in isolated bile canaliculi put directly on a cover glass was the enrichment of the lyn kinase in the cell-to-cell AJ immunofluorescently shown. As shown in Fig. 11, the lyn kinase was weakly but clearly detected to be colocalized with tenuin (49), one of the major constituents of the undercoat of the isolated AJ.

Discussion

The cell-to-cell AJ is the site of cell recognition and adhesion. Evidence has accumulated that the cell-to-cell adhesion molecule in AJ is cadherin and that AJ plays a crucial role in the tissue morphogenesis through cadherin and its associated proteins (6, 11, 14, 38, 46). Therefore, for a better understanding of the molecular bases of the tissue morphogenesis, it is necessary to analyze the signaling pathway in AJ. Set against this situation, in this study we have obtained the following results. (*a*) When the tissues of adult rats are pretreated with sodium orthovanadate (44), anti-phosphotyrosine pAb specifically stains the undercoat of cell-to-cell AJ of rat adult tissues. (*b*) In vitro autophosphorylation experiments using isolated AJ reveal that major tyrosine kinases in the cell-to-cell AJ of hepatocytes are c-yes, c-src, and lyn kinases. (*c*) At least two of these kinases, c-yes and c-src kinases, are enriched at the cell-to-cell AJ of various types of cells.

Recently, by the use of anti-phosphotyrosine pAb, Takata and Singer clearly showed that the phosphotyrosine-modified proteins were concentrated at the undercoat of cell-to-cell AJ during tissue development in chick embryos (45), and that this enrichment was not detected in the adult tissues. We performed the same experiments using rats and confirmed their results. However, as shown in this study, when the tissues were pretreated with sodium orthovanadate, a potent inhibitor of tyrosine phosphatase, anti-phosphotyrosine pAb clearly stained the cell-to-cell AJ of various types of adult

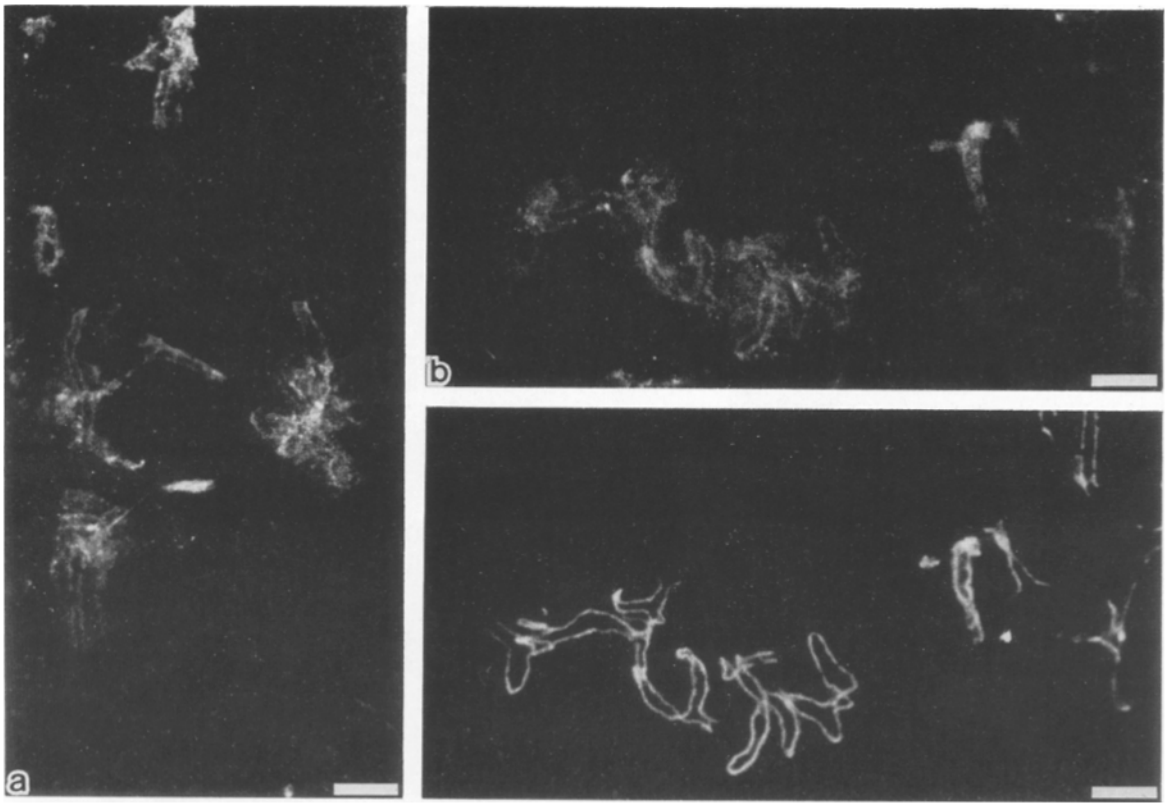

Figure 11. Immunofluorescence localization of the lyn kinase. (*a*) Isolated bile canaliculi were stained with anti-lyn pAb (*a*), or doubly stained with anti-lyn pAb (*b*) and anti-tenuin mAb (*c*). The lyn-kinase is localized in the cell-to-cell AJ that are labeled with anti-tenuin mAb (49). Bars, 1 μm.

tissues. There may be two possible explanations for this result. (*a*) The activity of tyrosine phosphatase may be higher in the adult tissues than in the embryonic tissues, so that in the absence of sodium orthovanadate most of the phosphates on tyrosine residues in AJ of adult tissues may be released during the procedures for immunofluorescence microscopy. (*b*) There may be a rapid turnover of phosphates on tyrosine residues in AJ of adult tissues, and the inhibition of phosphatase by sodium orthovanadate may result in the accumulation of phosphotyrosine-modified proteins at AJ. It is premature to discuss these possibilities further. However, considering that sodium orthovanadate can inhibit the activity of tyrosine phosphatases not only at AJ but also at the other regions equally, we can conclude that the tyrosine kinase activity is higher at the undercoat of cell-to-cell AJ as compared to the other regions of cells in adult tissues. Taken together with the previous results that in various types of cultured cells AJ was labeled with the anti-phosphotyrosine antibody (31), these findings enable us to conclude that tyrosine phosphorylation is used as an important signal at the cell-to-cell AJ.

In the present study, we have shown that two specific members of src family tyrosine kinases, c-yes and c-src kinases, are enriched specifically at the cell-to-cell AJ in various types of cells. The concentration of lyn kinase, another member of the src family, at the cell-to-cell AJ was detected only in hepatocytes. The c-yes and c-src kinases are ubiquitously expressed (8, 40, 41), while lyn kinase is expressed specifically in hematopoietic cells including B-lymphocytes (54, 55). Therefore, it may be reasonable to say that c-yes and c-src kinases are major tyrosine kinases generally responsible for the signaling at the undercoat of the cell-to-cell AJ in various types of adult tissues. Recent studies have clarified the localizations and functions of some src-family kinases. In neurons that lack a typical cell-to-cell AJ, the c-src kinase was shown to be concentrated in growth cone membrane (32) and in axon terminals (20), suggesting that it might play a role in signal transduction in neural cell recognition (8). In some types of cells, c-src kinase was reported to be associated with gap junctions and to regulate their functions (cell–cell communication) (1–3, 9). The lck and fyn kinases were reported to be associated with CD4/CD8 and T cell receptor (39, 51), respectively, and to be involved in the signal transduction of cell-cell recognition of T-lymphocytes. The lyn and blk kinases were supposed to be key signal transducers in B-cell activation (13, 55). Taking these results together with our observations, it is tempting to imagine that the proto-oncogenic kinases of the src family might play crucial roles in different types of cell recognition/communication in various types of cells, and that, in cells bearing the cell-to-cell AJ c-yes and c-src kinases might work as major signal transducers in AJ. The significance of coexistence of two distinct src-like kinases (in the case of hepatocytes, three kinases) at the undercoat of the cell-to-cell AJ is not clear at present. Is it a redundancy for the sake of safety? Does each kinase have its own specific function? The production of a mutant mouse lacking c-src and/or c-yes kinase using embryonic stem cells will give us a clear answer in the near future.

Our present study showing the enrichment of two src-like kinases at AJ gives us a hint regarding the possible molecular

mechanism of cell transformation induced by v-src products. It is widely accepted that the ability of v-src kinase to bind the plasma membrane is required for its transformation activity and that v-src kinase is highly concentrated in AJ of transformed cells (10, 15, 17, 18, 27, 36); concomitantly, AJ was seen to be modified or destroyed (26, 53). Therefore, it is interesting to imagine that v-src kinase accumulated in AJ may override the c-yes and c-src kinase-dependent signal transduction system in normal AJ to transform cells.

Since two specific src-like tyrosine kinases were identified in cell-to-cell AJ in the present study, the question naturally arises: How do these kinases work in signaling in AJ at the molecular level? To answer this question, two problems remain to be solved in future. What kind of transmembranous proteins anchor these src-like kinases to membrane proper at AJ and mediate extracellular signals to these kinases? What are the target substrates of these tyrosine kinases? To approach such AJ-specific protein–protein interactions, the isolated cell-to-cell AJ offers an advantageous in vitro system.

In this study, we have focused our attention on the tyrosine phosphorylation in the cell-to-cell AJ. Of course, there are possibilities that other kinds of signaling or cross-talking between the signaling pathways may also occur in AJ: specific types of protein kinase-C (24) and calcium-dependent protease (4) have been reported to be localized in only the cell-to-substrate AJ as candidates for signal mediators. The isolated AJ will again provide a useful model system for detailed analyses of these signal mediators in the cell-to-cell AJ. Further analyses on the signaling pathway in AJ using this system will lead to a better understanding of the molecular bases of tissue morphogenesis and carcinogenesis.

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