Specific intercellular binding of the β-amyloid precursor protein to the presenilins induces intercellular signaling: Its significance for Alzheimer's disease

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ABSTRACT Genetic evidence has implicated three proteins, the β-amyloid precursor protein (β-APP) and the two homologous presenilins (PS-1 and PS-2), in the etiology of Alzheimer's disease (AD). How these three proteins jointly contribute to AD, however, is not clear. Nor is any of their normal physiological functions known. Herein, we demonstrate, confirming a prediction made earlier, that β-APP and either PS-1 or PS-2 act as a specific membrane-bound ligand binding intercellularly with either of its two membrane receptors. This results in a cell–cell adhesion, after which rapid transient increases in protein tyrosine kinase activity and protein tyrosine phosphorylation occur coordinately inside one or both of the two adherent cells. The spectrum of proteins modified by tyrosine phosphorylation differs depending on whether PS-1 or PS-2 is involved in the specific intercellular binding to β-APP, which implies that PS-1 and PS-2 have distinct, rather than redundant, functions in normal physiology. The relevance of this intercellular interaction and signaling process to AD is discussed.

Most cases of Alzheimer's disease (AD) arise late in life and show only limited familial relationships. In approximately 10% of the cases, however, early onset forms of AD arise which are familial (FAD), involving single gene mutations that are autosomal dominant. Among the many individual cases of FAD, mutations in one of only three genes have been reported. These are the genes encoding the β-amyloid precursor protein (β-APP) (for a review, see ref. 1) and the two homologous presenilin proteins PS-1 (2) and PS-2 (3, 4). β-APP is the protein from which the β-amyloid (Aβ) oligopeptides are proteolytically derived (5). Aβ is found in the neuritic plaques in the brain that are characteristic of AD and FAD victims and is thought to be directly involved in the pathology of these diseases. In addition to β-APP, the genetic results with FAD families also strongly imply a crucial role for the wild-type presenilins in AD, but the nature of this role is not known. Furthermore, the normal physiological functions of either β-APP, PS-1, or PS-2 are yet to be defined. All three proteins are integral proteins of membranes.

We have proposed a mechanism (6) that involves the presenilins directly and specifically in the events leading to the proteolysis of β-APP to form Aβ. In this proposal, β-APP on the one hand and PS-1 (or PS-2) on the other are related as a specific membrane ligand on one cell surface to either of its two membrane receptors on a second cell surface. The specific intercellular binding of β-APP to either PS-1 or PS-2 would lead to a cell–cell adhesion (7); this was indeed subsequently demonstrated to occur (8). It was proposed that this specific adhesion could have different consequences, one normal and

the other pathological. The latter consequence would lead to the proteolysis of β-APP to form Aβ (see *Discussion*), whereas the former would involve an intercellular signal transmitted into one or both of the adhering cells specifically via the β-APP:PS binding. This signaling event would presumably reflect the normal physiological functions of the β-APP and PS membrane proteins, which perhaps triggers some particular developmental responses in the organism.

In the present study, we have sought evidence for this proposed β-APP:PS-mediated intercellular signaling event. Cultured cells that were transiently transfected with either β-APP, PS-1, or PS-2, were appropriately mixed, and were analyzed over time for protein tyrosine kinase activity, and for net protein tyrosine phosphorylation, by using antibodies specific for phosphotyrosine (PTyr). Within several minutes after mixing the β-APP-transfected cells with either the PS-1- or PS-2-transfected cells, the cell extracts showed significant transient increases in protein tyrosine kinase activity, and in PTyr modification of protein substrates, that did not appear in controls. Furthermore, these increases in tyrosine kinase activity and in PTyr formation were distinguishable between PS-1 and PS-2. These results provide strong support for our proposal (6) that a specific β-APP:PS-mediated intercellular interaction occurs, which is presumably involved in normal physiological responses. The results also are consistent with our proposed intercellular interaction mechanism (6) for the formation of Aβ from β-APP in the etiology of AD and familial AD (FAD).

MATERIALS AND METHODS

Cell Culture and Transfections. Human DAMI cells (American Culture Collection, CRL 9792) were cultured and transiently transfected with high efficiencies (60–80%) with pcDNA3 constructs of full length cDNAs for β-APP, PS-1, and PS-2, as described (8).

Antibodies. Affinity-purified polyclonal rabbit anti-PTyr antibodies (9) were used in all Western blots. Its specificity was demonstrated in anti-PTyr-labeling experiments carried out in the presence of 10 mM PTyr. A mouse monoclonal anti-PTyr antibody (PY20; Calbiochem) was used in enzyme-linked immunosorbent assay (ELISA) analyses. Three polyclonal rabbit anti-peptide antibodies specific for PS-1 were used (10, 11), two directed to two sequences within the large extracellular loop between transmembrane domains 6 and 7 (designated antibodies L1 and L3 in refs. 10 and 11), and one directed to a sequence in the extracellular NH₂-terminal domain (designated antibody N2 in refs. 10 and 11). One polyclonal rabbit anti-peptide antibody to PS-2 was used that

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Abbreviations: β-APP, β-amyloid precursor protein; AD, Alzheimer's disease; ELISA, enzyme-linked immunosorbent assay; FAD, familial AD; PS-1 and PS-2, presenilins 1 and 2; PTyr, phosphotyrosine; Aβ, β-amyloid.
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was directed to an NH_2-terminal domain sequence (designated N1 in refs. 10 and 11). A horseradish peroxidase conjugate of goat anti-rabbit IgG was obtained from Bio-Rad.

Cell Mixing and Extractions. Equal numbers (0.5×10^6/ml) of β-APP-transfected DAMI cells and either PS-1- or PS-2-transfected DAMI cells were mixed gently at room temperature. In control experiments, DAMI cells transfected with pcDNA3 alone were substituted for the β-APP-transfected cells. At several times between 0 and 20 min after mixing, one aliquot of the cell mixture was rapidly centrifuged, the culture medium was removed, and the cell pellet was suspended in 200 μl of extraction buffer (50 mM Tris, pH 8.0/150 mM NaCl/ 0.5% Nonidet-40) containing protease inhibitors (1 μM phenylmethylsulfonyl fluoride/1 μg/ml antipain/0.1 μg/ml pepstatin A/0.1 μg/ml leupeptin) and the phosphatase inhibitor 0.1 mM sodium orthovanadate. The mixture was sonicated with three bursts of 20 sec duration and then centrifuged. These extract supernatants were then used for the Western blot and ELISA analyses as described below. In some experiments, the intact PS-1- or PS-2-transfected cells were first reacted with anti-PS-1 or anti-PS-2 antibodies, respectively, for 15 min at room temperature before mixing with the β-APP-transfected cells. The anti-PS-1 antibodies used in this case were an equal-part mixture of the three antibodies L1, L3, and N2.

Determination of Cell Aggregation. Aliquots of the cell mixtures (100–200 μl) were removed at various times after mixing, immediately plated onto plastic tissue culture dishes (10 $\times$ 35 mm), and viewed under the light microscope.

Western Blotting. Aliquots of the extract supernatants described above (100 μg of protein/lane) were boiled for 5 min in loading buffer (50 mM Tris, pH 6.8/0.1 M DTT/2% SDS/0.1% bromphenol blue/10% glycerol), separated electrophoretically on SDS/PAGE (10%) gels, and the proteins transferred electrophoretically onto nitrocellulose filters. Filters were incubated with the primary polyclonal rabbit anti-PTyr antibodies followed by the horseradish peroxidase-conjugated goat anti-rabbit IgG. Filter-bound peroxidase activity was determined with 4-chloro-1-naphthol.

ELISAs. Protein tyrosine kinase activity was measured by an ELISA using a protein tyrosine kinase assay kit (Calbiochem). Plates were provided that were precoated with polymer tyrosine residues. Supernatants of extracts of cells mixed for different times (20 μg of protein/well) were added to the

precoated wells and allowed to phosphorylate the bound substrate after the addition of Mg^{2+} and ATP according to the manufacturer's instructions. A PTyr-specific mouse monoclonal antibody PY20 conjugated to horseradish peroxidase was used to detect the phosphotyrosyl residues at 450 nm.

RESULTS

ELISA Analyses for Protein Tyrosine Kinase Activity. The addition of β-APP-transfected DAMI cells to PS-1-transfected DAMI cells resulted in a transient increase in protein tyrosine kinase activity measured in extracts of the cell mixtures (Fig. 1*A*), which peaked at $\approx$8–10 min after mixing and returned to baseline values by 12–14 min. The substitution of cells transfected with pcDNA3 alone for the β-APP-transfected cells in these experiments (Fig. 1*C*) did not result in any such increase in kinase activity. If the PS-1-transfected cells were first treated with the combined anti-peptide antibodies to PS-1 for 15 min, and were then mixed with the β-APP-transfected cells, no increase in protein tyrosine kinase activity (Fig. 1*D*) was observed. (In these experiments, the zero time in Fig. 1*D* refers to the time the two cell populations were mixed. Assays at earlier times (not shown) of the antibody-treated PS-1-transfected cells before mixing with the β-APP-transfected cells showed no kinase activity above baseline.) These results demonstrate that specific antibody blocking of the PS-1 molecules at the cell surface prevents the increase in protein tyrosine kinase activity in the transfected cell mixtures.

The mixing of β-APP-transfected cells with PS-2-transfected cells also resulted in a transient increase in protein tyrosine kinase activity (Fig. 1*B*), but the time sequence was different from that observed when the PS-1-transfected cells were used. Two peaks of activity were observed at $\approx$8 and 18 min after cell mixing, and by $\approx$20 min, the activity levels returned to baseline values. If anti-PS-2 antibodies were first incubated for 15 min with the PS-2-transfected cells before adding the β-APP-transfected cells, the increases in protein tyrosine kinase activity that were observed in the absence of the antibody treatment were not exhibited (Fig. 1*E*).

Western Blotting Experiments. The same or similar extracts used for the ELISA experiments also were subjected to SDS/PAGE and immunoblotting with anti-PTyr antibodies to detect the time course of any net PTyr modification of specific cell proteins. Two sets of immunoblots of extracts from
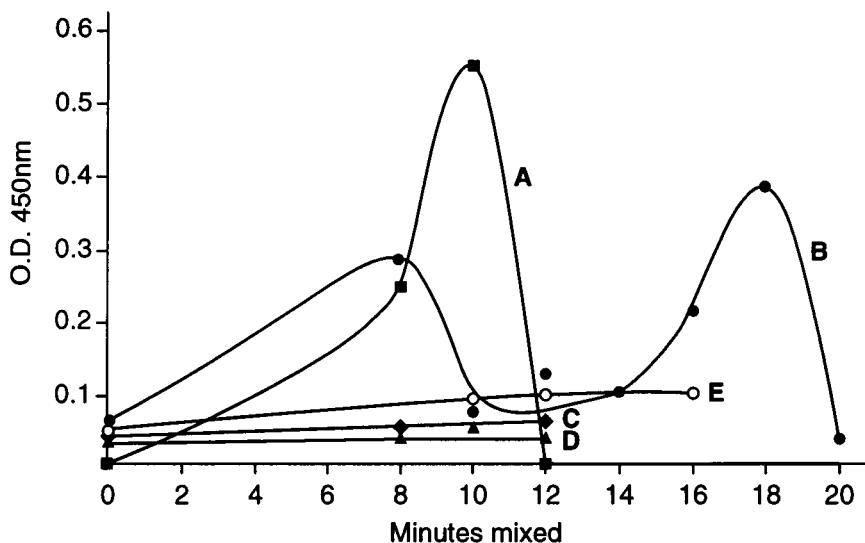

FIG. 1.   ELISA experiments measuring the protein tyrosine kinase activity as a function of time after the mixing of two appropriately transfected DAMI cell populations. β-APP-transfected cells with PS-1-transfected cells (*A*); β-APP-transfected cells with PS-2-transfected cells (*B*); pcDNA3-(vector only) transfected cells with PS-1-transfected cells (*C*); PS-1-transfected cells (*D*), or PS-2-transfected cells (*E*), first treated with specific anti-PS-1 or anti-PS-2 antibodies, respectively, before mixing with β-APP-transfected cells.

independent experiments with mixtures of β-APP-transfected cells and PS-1-transfected cells are shown in Fig. 2 *A* and *B*. These two experiments were chosen because they indicate the reproducibility of the principal features of the results, although certain minor details are somewhat variable. The immunoblots in Fig. 2 *A* and *B* show a transient increase in protein PTyr modification that peaks at ≈8–10 min., closely similar to the increase in protein tyrosine kinase activity in Fig. 1*B*. Two main protein bands of apparent molecular masses 58 and 60 kDa undergo the transient modification. If cells transfected with the vector alone are used instead of the β-APP-transfected cells, no significant increase in PTyr modification is observed in the same time interval (Fig. 2*C*). The presence of an excess of PTyr (Fig. 2*D*) inhibits the immunoblotting observed in Fig. 2 *A* and *B*, attesting to the specificity of the anti-PTyr antibodies that were used.

Similar immunoblotting experiments with extracts from two independent cell mixtures of β-APP-transfected cells and PS-2-transfected cells (Fig. 3 *A* and *B*) again show a transient increase in net protein tyrosine phosphorylation but with several distinct differences from the results when the PS-1-transfected cells were involved (Fig. 2 *A* and *B*). Among these characteristic differences were: a longer time span for the transient PTyr-modification of proteins in the PS-2 than in the PS-1 case, as also was seen in the ELISA results for the kinase activity (Fig. 1*B*); and an entirely different set of proteins modified with PS-2 than with PS-1. In the PS-2 case, a major protein band of 51 kDa, additional bands at 53, 55, 57, and 66 kDa, a minor doublet at ≈48 kDa, and a triplet at 32–34 kDa all underwent transient PTyr modification. [The minimum observed in the ELISA kinase activity experiments of Fig. 1*B* is not readily apparent in Fig. 3 *A* and *B* but was clearly visible at early stages in the development of the peroxidase color reaction (not shown)]. Substitution of cells transfected with the

vector alone for the β-APP-transfected cells showed no increase in PTyr modification in the same time span (Fig. 3*C*).

Cell Aggregation. We have shown earlier (8) that in mixtures of intact β-APP-transfected DAMI cells with either PS-1- or PS-2-transfected DAMI cells, the two kinds of cells aggregate with each other specifically. This constituted the first evidence for the specific molecular binding of β-APP with either PS-1 or PS-2, which has since been confirmed by others (12, 13). To compare the time course of this cell aggregation with the time course of the increase in protein tyrosine kinase activity and in protein tyrosine phosphorylation in the same preparations, aliquots of the mixtures of whole cells were examined by light microscopy for cell aggregation. A representative experiment is pictured in Fig. 4, column *A*. In mixtures of β-APP transfected with PS-1-transfected cells, significant cell aggregation was observed by 6 min after mixing (Fig. 4*A*3) and persisted thereafter (Fig. 4*A*4–*A*6). Not all single cells enter aggregates, which is most likely due to the only limited transfection efficiencies (60–80%) that were achieved. This cell aggregation, as had been demonstrated earlier (8), specifically required the mediation of β-APP because mixtures of cells transfected with the pcDNA3 vector alone with PS-1-transfected cells showed no significant aggregation. The results show the specific cell aggregation is first clearly evident (Fig. 4*A*, 3) at approximately the same time as the increase in protein tyrosine kinase activity (Fig. 1*A*) and PTyr modification (Fig. 2) occurs.

Having shown (Fig. 1*D*) that specific antibodies to PS-1 block the increase in protein tyrosine kinase activity in mixtures of β-APP-transfected cells with PS-1-transfected cells, we next examined the extent of cell aggregation in the same antibody-cell mixtures. Immediately (zero time) after the transfected cells were mixed, cell aggregates were observed (not shown). This aggregation led us to suspect that the bivalent anti-PS-1 antibodies were not simply blocking PS-1
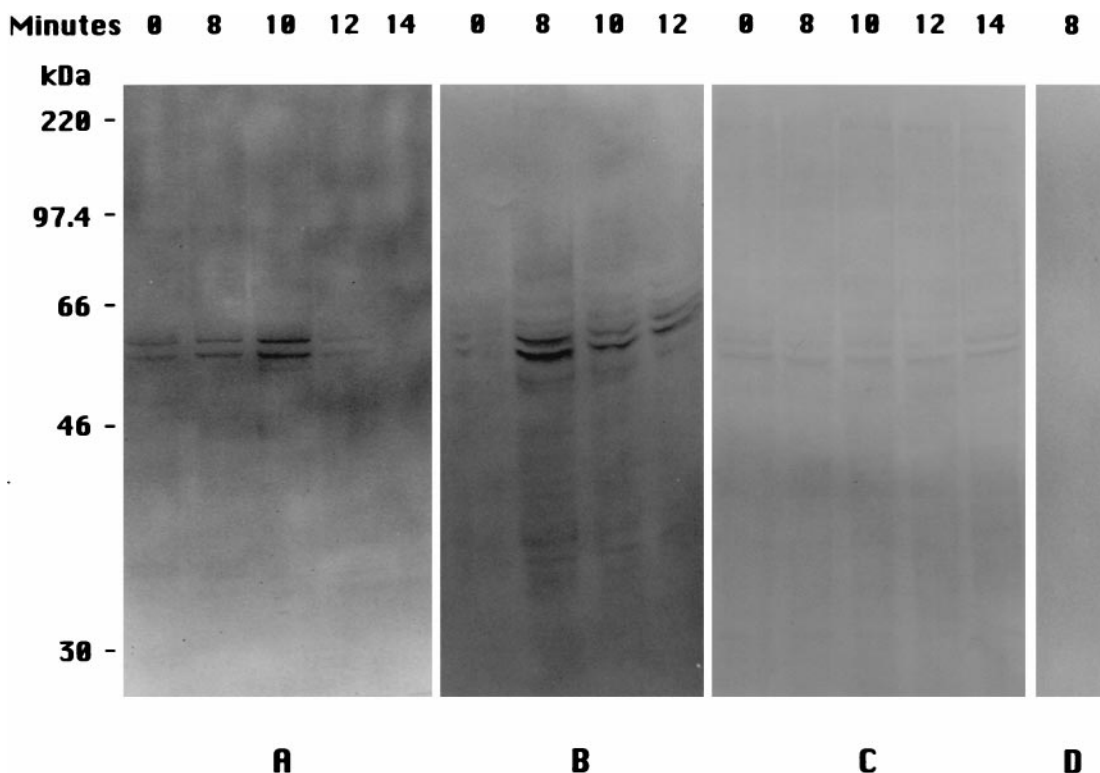

FIG. 2. Western immunoblots with primary anti-PTyr antibodies of extracts from two independent experiments (*A* and *B*), in which β-APP-transfected cells were mixed with PS-1-transfected cells and the mixtures sampled at different times after mixing. (*A* and *B*) The basic similarity and minor variability observed in replicates of these experiments. (*C*) Similar experiments in which pcDNA3-transfected cells were used in place of the β-APP-transfected cells. (*D*) Specificity test of the anti-PTyr antibodies used. The antibody labeling of the 8-min sample in *B* was carried out in the presence of excess free PTyr.
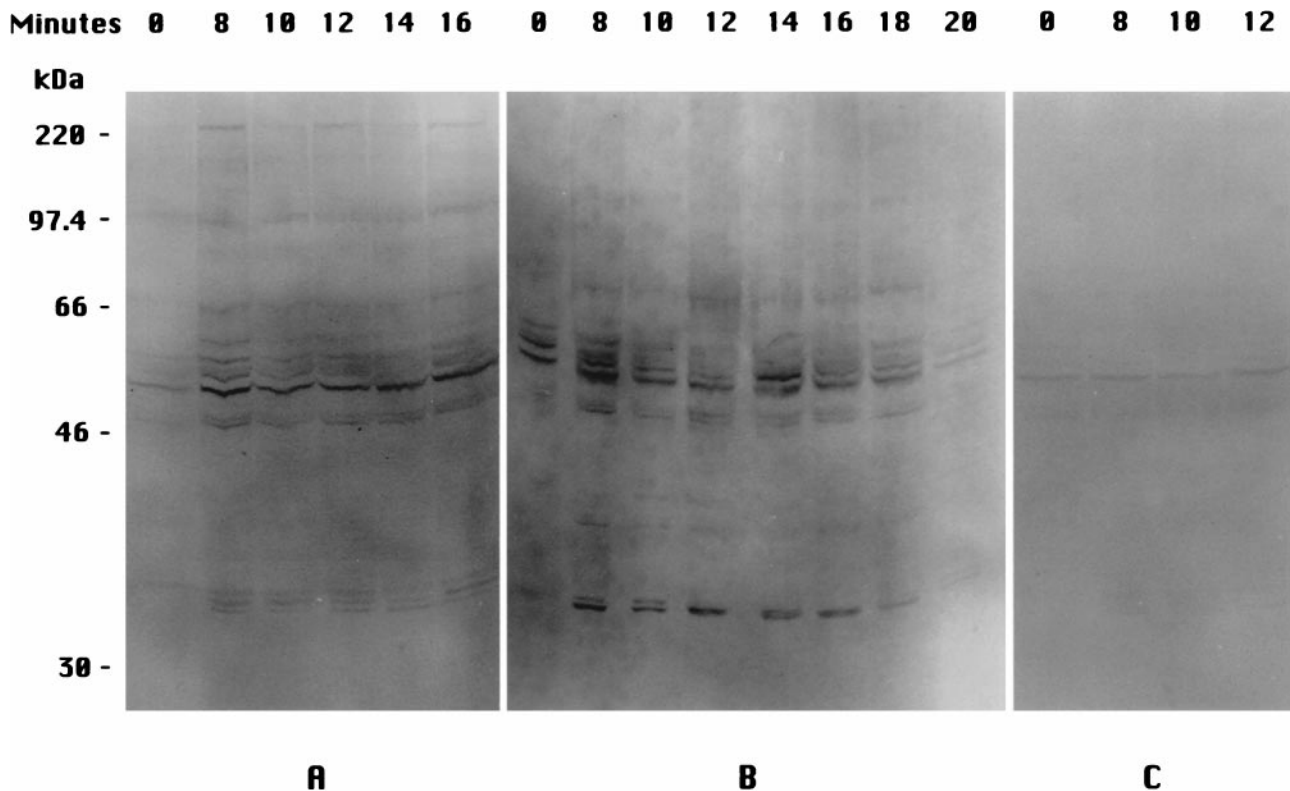
FIG. 3.   (*A–C*) Same as Fig. 2 *A–C*, respectively, except that PS-2-transfected cells were used instead of PS-1-transfected cells. Note that the PTyr- modified protein bands in *A* and *B* differ from those in Fig. 2*A* and *B*.

sites, but were cross-linking PS-1 molecules between two cells, causing the cells to adhere even in the absence of the β-APP-transfected cells. This suspicion was confirmed by examining the effects of anti-PS-1 antibodies on the PS-1-transfected cells alone (Fig. 4, column *B*). [DAMI cells do not express significant amounts of endogenous β-APP (8, 14)]. By 2 min after antibody addition, the PS-1-transfected cells had begun to aggregate (Fig. 4*B*3). Under the same conditions, but in the absence of the antibody, these cells show no significant aggregation (Fig. 4, column *C*).

DISCUSSION

Ever since molecular genetic studies of patients with FAD revealed the previously unrecognized existence of the presenilin proteins, and their likely critical importance, along with β-APP, to the pathology of AD, several questions have been raised but have gone unanswered. What is the functional relationship between β-APP and the PS proteins? What is the role of the PS proteins in the pathology of AD? What are the normal physiological functions of the ubiquitous β-APP and PS proteins? In this paper, we have provided evidence bearing directly on the first and third questions, and indirectly on the second, all consistent with the answers to these questions that we have earlier proposed (6).

When β-APP-transfected DAMI cells are suspended with PS-1- or PS-2-transfected DAMI cells, a specific cell–cell adhesion and cell aggregation occur (8; Fig. 4, column *A*), which require the cell surface expression of both β-APP and of either PS-1 or PS-2, and is inhibited by an excess of soluble fragments of the β-APP molecule (8). This cell–cell adhesion is therefore mediated by the specific transcellular binding of β-APP on the surface of one cell with either PS-1 or PS-2 on the surface of the other. We have herein shown that this specific adhesion leads to a rapid and transient increase in protein tyrosine kinase activity and protein tyrosine phosphorylation in most likely one, or possibly both, of the adhering

cells. (We do not yet know which is the case.) Clearly, the close parallelism between the two sets of results suggests that these two assays measure sequential aspects of the same signal induction process. If cell–cell adhesion and cell aggregation is induced instead by the antibody-induced cross-linking of PS-1 molecules on the PS-1-transfected cells (Fig. 4, column *B*), no increase in protein tyrosine kinase activity is observed with or without the subsequent addition of β-APP-transfected cells (Fig. 1*D*). Therefore, neither the engagement of the PS-1 molecules at the cell surface *per se*, nor the formation of a cell–cell adhesion *per se*, is responsible for the induction of the increase in protein tyrosine kinase activity; the evidence is compelling that the induction of this transient increase in kinase activity requires the specific cell–cell adhesion mediated by the intercellular binding of β-APP to either PS-1 or PS-2.

Of particular interest is that the downstream consequences of this signaling are different depending on whether PS-1 or PS-2 is engaged in the intercellular binding to β-APP, because the spectrum of proteins that show enhanced tyrosine phosphorylation is altogether different in the two cases (Figs. 2 and 3). This report is experimental evidence for a distinction between, rather than a redundancy of, the biochemical functions of the two closely homologous PS proteins. The significance of the two peaks of kinase activity at 8 and 18 min in the β-APP:PS-2 transient-signaling process (Fig. 1*B*) is not clear; the same spectrum of proteins appears to be PTyr modified throughout this time period. It is possible, for example, that a tyrosine phosphatase activity also is transiently induced, which has a peak at ≈12 min, but we have no evidence on this point.

Protein tyrosine phosphorylation is a well-known biochemical response that is common to many different ligand-receptor-mediated signaling processes (15), and the central nervous system is particularly rich in protein tyrosine kinases (cf. 16). The increase in PTyr is usually an early and transient postsignaling event, often initiated within a few minutes of ligand-receptor binding. Our results on protein tyrosine kinase
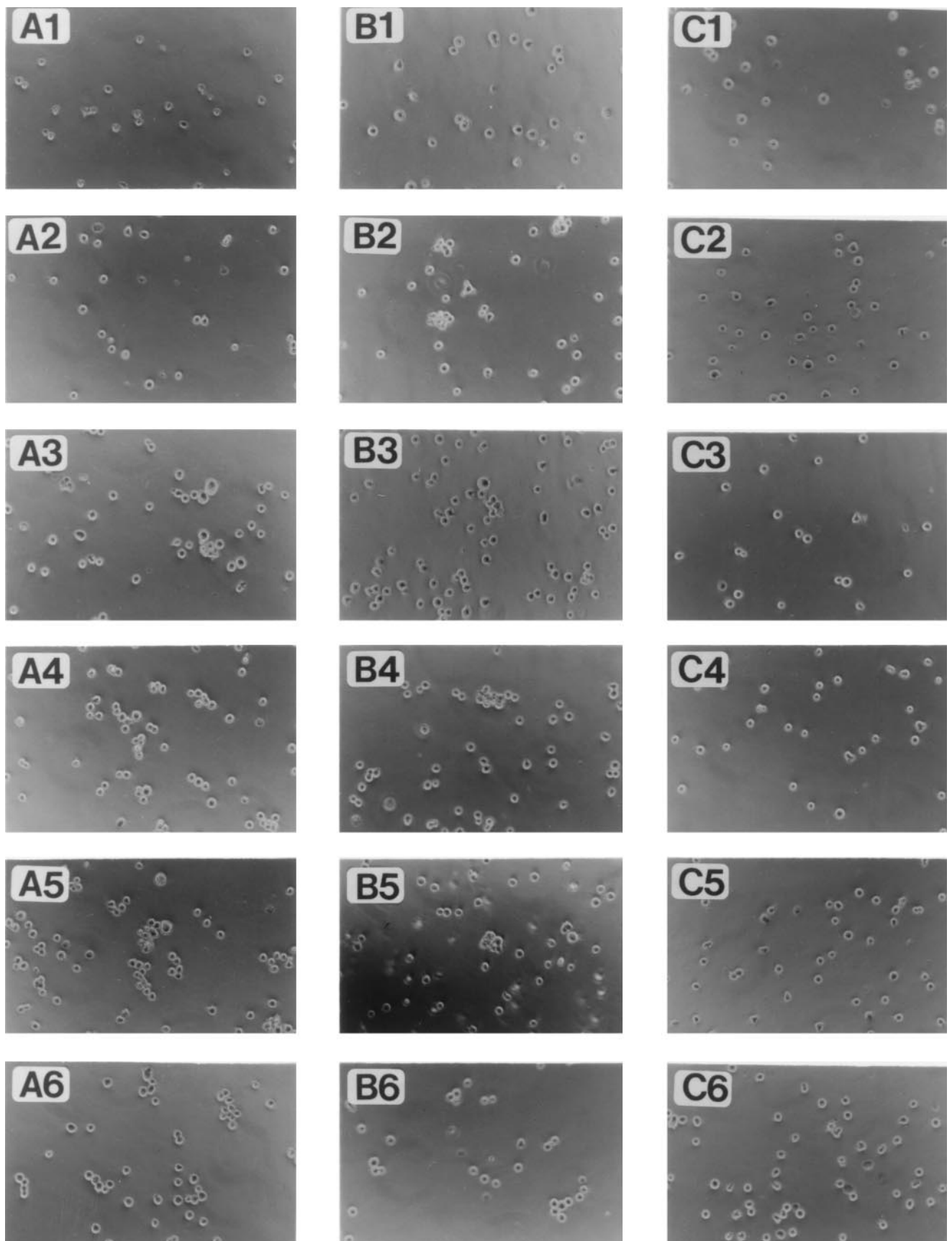
FIG. 4.   Light microscopic observation of cell aggregation as a function of time after mixing cells and/or reagents: column *A*, β-APP-transfected cells with PS-1-transfected cells; column *B*, PS-1-transfected cells mixed with anti-PS-1 antibodies, in the absence of β-APP-transfected cells; column *C*, PS-1-transfected cells alone. In all columns, the rows 1–6 represent the times 2, 4, 6, 8, 10, and 12 min, respectively, after mixing. In column *A*, the β-APP-transfected cells and PS-1-transfected cells began to show significant aggregation by 6 min (*A*3); in column *B*, the anti-PS-1 antibodies caused the PS-1-transfected cells to aggregate by 4 min (*B*2); in column *C*, no aggregation occurred.

activity increases and PTyr modification increases with the β-APP:PS mediated cell–cell interactions therefore are similar to those observed in many other signaling systems. Neither β-APP nor the PS proteins, however, are protein tyrosine kinases, and the kinases responsible for the PTyr modification in these systems are not known. It may be significant, however, that the cytoplasmic region of the β-APP molecule contains a domain with demonstrated binding affinity for PTyr-binding domains on certain other cytoplasmic proteins (17–20). This affinity may play a role in the downstream effects of an early PTyr protein modification event in β-APP:PS signaling.

We presume that the physiological function of the cellular signaling processes that we have demonstrated, as in many other cases (15), is to initiate one or a set of developmental changes in the organism. This signaling may be related to the *in vivo* consequences observed upon knockout of the PS-1 gene in (PS$^{-/-}$) mice (21, 22). Such mice die shortly after birth, and late embryos show impaired neurogenesis and marked skeletal defects. These developmental defects in PS$^{-/-}$ mice imply that PS-1 is required for some molecular activities leading to normal embryonic development.

All of these studies were carried out to increase our understanding of the etiology of AD, and our findings are of significance to that understanding. In our original paper (6), we postulated that our proposed β-APP:PS mediated cell–cell adhesion might involve particular neurons and perhaps other cells in the brain. We suggested that, as a by-product of this cell–cell adhesion, a special endocytic process was stimulated, in which double membrane-bounded vesicles, containing intact β-APP and PS molecules still bound to one another, would be pinched off the adherent cell surface of the β-APP-expressing cell and be taken into its interior. These vesicles would then fuse with multivesicular bodies inside the cells, whose resident proteases would break down the engulfed β-APP, forming Aβ. The usual intracellular traffic between the lysosomal compartment and the plasma membrane would then release the Aβ to the cell exterior, where it would accumulate in the neuritic plaques. Precedents for this unusual type of endocytosis are known (23, 24). Our proposal therefore is that the formation of the Aβ that is relevant to AD obligatorily involves an as yet unspecified pair of cells in the brain first adhering to one another through the specific binding of β-APP to one of the PS proteins. In the present paper, we have demonstrated that not only does such a β-APP:PS mediated cell–cell adhesion occur, but that it has functionally significant consequences, e.g., intercellular signaling. It remains to determine whether this specific adhesion also results in the enhanced production of the forms of Aβ that are relevant to AD.

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