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Antibody-induced linkages of plasma membrane proteins to intracellular actomyosin-containing filaments in cultured fibroblasts

(transmembrane linkages/membrane protein mobility/immunofluorescence/actin and myosin in non-muscle cells)

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ABSTRACT **The surface distributions of three different membrane integral proteins, β_2-microglobulin (part of the histocompatibility antigen complex), aminopeptidase (α-aminoacyl-peptide hydrolase; EC 3.4.11.2), and the Na^+,K^+-ATPase (ATP phosphohydrolase; EC 3.6.1.3) on human fibroblasts grown in monolayer culture have been studied with their specific antibodies by immunofluorescence. On the same cells, the distribution of intracellular actin was observed by a spectrally distinct fluorescent staining procedure. If each of the antibody reagents was permitted to cluster its specific protein in the plane of the membrane, these clusters apparently became linked, through the membrane, to actin- and myosin-containing filaments (stress fibers) underneath the membrane, and were thereby immobilized. From these and other experiments, it appears that most, if not all, integral proteins can, upon clustering, form such transmembrane linkages to actin and myosin. A molecular mechanism for the formation of these linkages is proposed which postulates that actin is associated with the cytoplasmic surface of plasma membranes by peripheral attachment to a ubiquitous integral protein X in the membrane; when other integral proteins are induced to form clusters, they become bound to X and hence to actin (and myosin). The possible physiological role of these transmembrane linkages is briefly discussed.**

The idea that cell surface molecules can interact with intracellular cytoskeletal proteins has been widely entertained (for reviews see refs. 1–3). Until recently, however, the primary evidence for this has been indirect, based on the effects of drugs such as cytochalasin B and colchicine on the lateral mobilities of cell surface molecules. Direct evidence for such transmembrane linkages, however, was provided by Ash and Singer (4). They showed by double-fluorescence staining experiments that when fluorescein-conjugated concanavalin A (Fl-Con A) was bound to the surfaces of normal rat kidney (NRK) cells in monolayer culture, the Fl-Con A was originally uniformly distributed over the surfaces, but after 20 min incubation at 37°, a substantial part of the Fl-Con A was lined up precisely over actin- and myosin-containing filaments that were present in more-or-less parallel arrays directly under the plasma membrane. The lining-up and transient immobilization of the Fl-Con A strongly suggested that physical linkages were produced between the Con A receptors in the membrane and the intracellular filaments. This linkage required a prior clustering of the Con A receptors by the tetravalent Fl-Con A, since treatment with divalent succinyl-Con A did not induce clustering or linkage of the receptors. Colchicine treatment did not affect the linkage phenomenon, indicating that microtubules played no direct role in it.

These observations immediately raised questions about the generality, the molecular mechanisms, and the significance of such transmembrane linkages. In this paper, we have extended our experiments to several independent integral membrane proteins on the surfaces of human fibroblasts, including β_2-microglobulin (5) and the enzymes aminopeptidase (α-aminoacyl-peptide hydrolase; EC 3.4.11.2) (6) and Na^+, K^+-ATPase (ATP phosphohydrolase; EC 3.6.1.3) (7). Antibodies specific for these proteins were used to induce their clustering and to observe their surface distributions. In each case, a similar transmembrane linkage was induced. Furthermore, from separate studies* (8) of the well-known capping phenomenon, it appears that closely similar transmembrane linkages to actin and myosin are induced during the capping of a wide variety of surface receptors on lymphocytes and other cells. Taken together, these results attest to the generality of these transmembrane linkage phenomena. To explain them, we propose a molecular mechanism that is subject to experimental test. Finally, the possible physiological significance of these linkage effects is addressed.

MATERIALS AND METHODS

Cell Culture. WI-38 cells were obtained from the American Type Culture Collection (CCL 75). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics under a humid atmosphere of 90% air/10% CO_2 at 37°. Cells were plated onto glass coverslips at 2×10^4 cells per 35-mm dish 2 days prior to an experiment. Cells between the 25th and 30th doubling were used for these studies.

Antibodies and Staining Reagents. The primary antibodies to membrane proteins were goat antibodies against rabbit intestine aminopeptidase purified by affinity chromatography (H. Feracci and S. Maroux, unpublished), IgG of bovine antibodies against human β_2-microglobulin purified by DEAE-cellulose chromatography (9), and IgG of rabbit antibodies against dog kidney Na^+,K^+-ATPase holoenzyme purified by DEAE-cellulose chromatography (7). These reagents were generous gifts of S. Maroux and H. Feracci, R. Reisfeld, and J. Kyte, respectively. $(Fab')_2$ antibody fragments of the goat antibodies against rabbit aminopeptidase, bovine antibodies against human β_2-microglobulin, and rabbit antibodies against bovine IgG were prepared from IgG fractions by pepsin digestion and separated from undigested IgG and Fc fragments by gel filtration chromatography on Sephadex G-100. Secondary antibodies used for indirect immunofluorescent staining were goat antibodies against rabbit IgG, rabbit anti-

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Abbreviations: Con A, concanavalin A; Fl-Con A, fluorescein-conjugated Con A; NRK cells, normal rat kidney cells.

* L. Y. W. Bourguignon, K. T. Tokuyasu, and S. J. Singer, unpublished.

bodies against goat IgG, and rabbit antibodies against bovine IgG. These IgG fractions, purified by DEAE-cellulose chromatography, were conjugated with Lissamine Rhodamine (Polysciences, Inc., Warrington, PA) and repurified on DEAE-cellulose to obtain fractions with 2 mol of rhodamine per mol of IgG, as described by Brandtzaeg (10). The rhodamine conjugates were finally isolated by affinity chromatography.

Biotinyl-heavy meromyosin and fluorescein isothiocyanate-conjugated avidin (Fl-avidin) used to stain intracellular actin were prepared as described (11).

Cell Staining. Cells stained for initial surface membrane distributions were fixed with 3% formaldehyde in phosphate-buffered saline for 20 min before antibody incubations. Other cultures that were treated to obtain surface redistributions were rinsed with serum-free medium and incubated with the primary antibody or $(Fab')_2$ fragments in this medium for 20 min at 37°. [The bovine antibodies against β_2-microglobulin, unlike the two other primary antibodies, did not itself redistribute its surface antigen and an additional 20-min incubation with rabbit antibodies against bovine IgG or $(Fab')_2$ fragment was used in this case.] After this incubation the cells were rinsed and fixed. In all cases, after fixation the cells were rinsed and treated with 0.1 M glycine in phosphate-buffered saline and stained indirectly with two successive layers of rhodamine-conjugated antibodies. The use of two layers of fluorescent antibodies was necessary to amplify the signal arising from these membrane proteins, which were relatively sparsely present on the surface. Controls utilized the appropriate normal IgG in place of the primary antibody and uniformly yielded only low background staining. After the surface staining the cells were processed for the localization of intracellular actin as described (12). For these experiments actin localization with biotinyl-heavy meromyosin was very useful because, not involving any antibody reagents, it did not interfere with the membrane antibody-staining systems. Cells were photographed on Kodak Plus X film with a Zeiss Photomicroscope III with epifluorescent illumination and specific interference filters for fluorescein and rhodamine (12). The filter systems were changed without altering the focus, which was usually set at the upper or lower cell surface, as indicated in the figure legends. The Plus X film was developed with Kodak Microdol (1:3) and enlarged onto Kodak fine grain positive film 7302 to produce transparencies useful for careful comparison of the distributions of fluorescein and rhodamine fluorescence.

RESULTS

The surface distributions of each of the three membrane proteins were examined with the fluorescent antibody reagents under two conditions: (*i*) on fixed cells, which we expected would reveal the initial distribution of the proteins in the plane of the membrane; and (*ii*) on cells incubated with the antibody reagents to induce clustering of the membrane protein before the cells were fixed. Only results for the aminopeptidase and β_2-microglobulin proteins are shown. The results for the Na^+,K^+-ATPase appeared closely similar in the fluorescence microscope, but the fluorescence was too weak for suitable photography.

The *initial* distributions of these surface proteins were, surprisingly, not uniform. With the appropriate intensity of fluorescent labeling and by careful focusing on the cell surfaces, the fluorescence was seen to be finely punctate (Fig. 1 *B* and *F*). Furthermore, on regions of the cell surface lying immediately over stress fibers, the tiny dots of surface fluorescence were arranged in linear arrays that were parallel to the linear arrays of stress fibers that were observable in the same focal plane. The two kinds of linear arrays were not overlapping, however; instead, superimposition of the positive transparencies showed that *in projection* the arrays were interdigitated. The nonoverlapping of the linear arrays was also evident in regions where the stress fibers came together in a cusp-like configuration (c in Fig. 1 *A* and *E*); in the membrane immediately over such cusps the surface fluorescence was absent (c in Fig. 1 *B* and *F*), which would not be the case if the two kinds of linear arrays did overlap.

The initial distributions of the surface proteins were not, however, entirely in such linear arrays. On regions of the cell surface that did not exhibit underlying stress fibers, as in ruffles (not shown), the tiny dots of surface fluorescence appeared to be randomly distributed.

In contrast to this punctate and largely linear initial distribution of aminopeptidase and β_2-microglobulin, the initial distribution of the fluorescence of Fl-Con A bound to NRK cells (4) or to these same human fibroblasts (not shown) was completely uniform within the resolving power of the microscope.

The distribution of each of the membrane proteins, after clustering had been induced in the cell surface by the specific antibody reagents, was markedly different from the initial distribution. First, the spots of surface fluorescence were now much larger (Fig. 1 *D* and *H*). Second, although these spots were again arranged in linear arrays that were parallel to the arrays of stress fibers, the two kinds of arrays were now clearly superimposable rather than interdigitated (compare Fig. 1 *C* and *D*; and *G* and *H*). These linear surface arrays were seen on essentially all of the cells so treated. These arrays formed whether IgG antibodies or their $(Fab')_2$ fragments were used, and were observed whether or not subsequent staining of the intracellular actin was carried out.

Another important difference between the initial distribution of the membrane proteins and the distribution induced after antibody clustering was evident on those areas of the surface that were involved in ruffle membrane activity and that were relatively free of immediately underlying stress fibers. On such areas (not shown) the aminopeptidase and β_2-microglobulin were found in one or two large patches, with adjoining large areas of the membrane completely free of fluorescence.

The distributions of membrane proteins seen in Fig. 1 *D* and *H* were attained in the presence as well as the absence of 20 mM NaN_3 (30-min pretreatment in glucose-free medium at 37°) or of 10 μM colchicine (3-hr pretreatment in medium at 37°).

DISCUSSION

In our previous study (4), we investigated the properties of the surface receptors for the lectin Con A on NRK cells. These receptors are probably a heterogeneous group of glycoprotein molecules that are not at present well characterized. By contrast, the membrane proteins studied in this paper are specific molecular species that have been isolated and investigated structurally. The β_2-microglobulin molecule is attached to the major histocompatibility antigen molecule (HLA) on human cell surfaces (13) (see, however, ref. 14) and aminopeptidase† (15) and Na^+,K^+-ATPase (7) are widely distributed on cell surfaces. Each of these proteins is an oligomeric molecule, part

† Aminopeptidase is also known as leucine aminopeptidase or L-leucyl-β-naphthylamidase and has been detected in a wide variety of tissues (16).

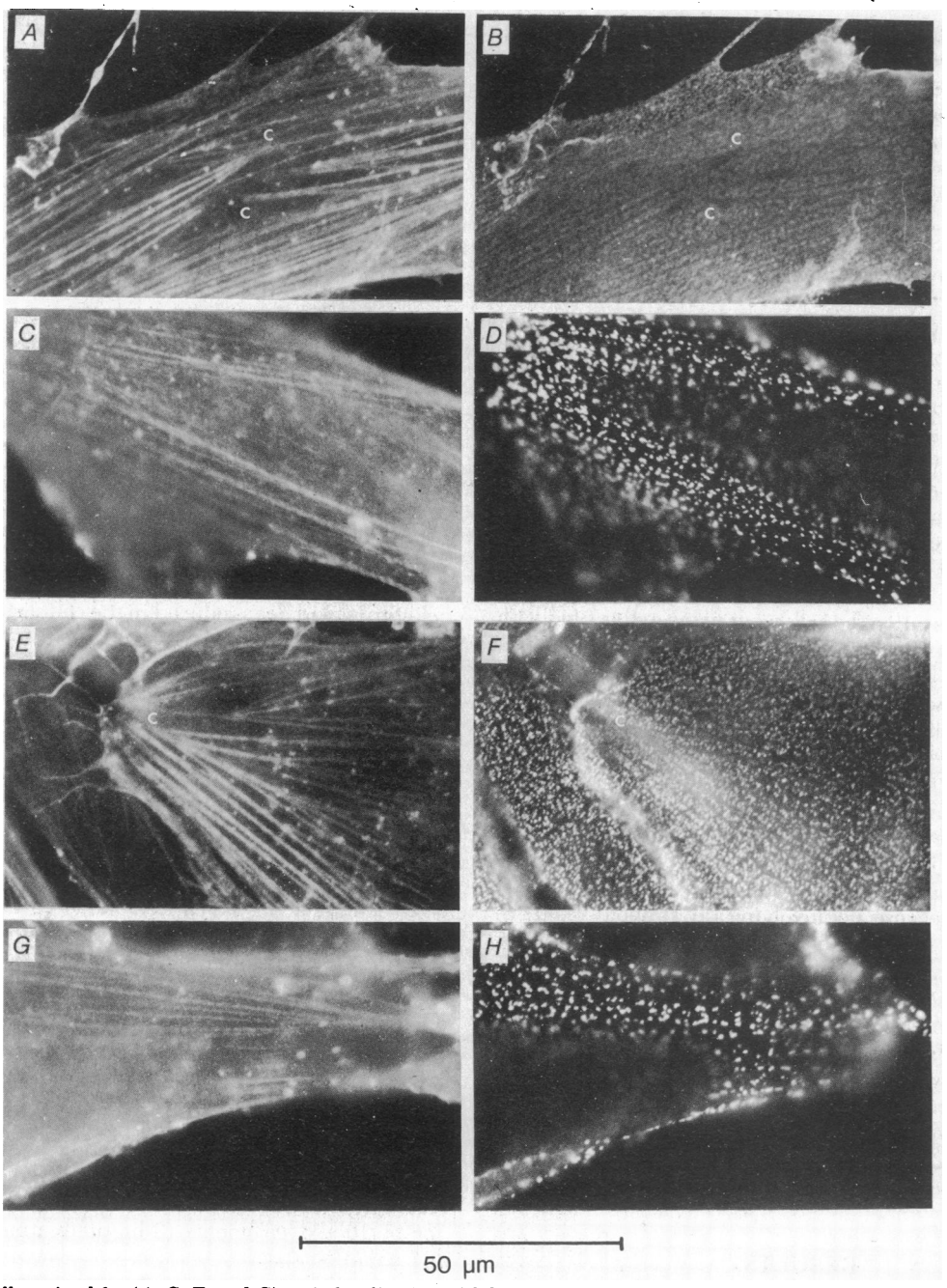

FIG. 1. WI-38 cells stained for (*A*, *C*, *E*, and *G*) actin localization with biotinyl-heavy meromyosin and Fl-avidin and simultaneously for (*B*, *D*, *F*, and *H*) membrane protein distribution by indirect rhodamine immunofluorescence. (*A* and *B*) The cell was fixed before staining to reveal the initial distribution of actin in *A* and β_2-microglobulin in *B*. The focus is at the bottom of the cell. Actin fibers in *A* converge to form cusps [two are labeled (c)], which correspond to large gaps in the β_2-microglobulin distribution in *B*. There are also linear gaps in the β_2-microglobulin distribution which correspond to actin fibers. (*C* and *D*) The cell was fixed after incubation with the $(Fab')_2$ antibody system, which produced a redistribution of the surface β_2-microglobulin. The focus is on the upper surface of the cell midway between the top and bottom. The patches of β_2-microglobulin in *D* are lined up over the submembranous actin fibers in *C*. (*E* and *F*) Initial distribution of actin in *E* and aminopeptidase in *F* on the bottom surface of a cell. As in *A* and *B*, actin cusps (c) and fibers correspond to gaps in the aminopeptidase distribution. (*G* and *H*) Redistribution of surface aminopeptidase by its $(Fab')_2$ antibody produces patches of fluorescence on *H* which line up over actin fibers in *G*. Focus as in *C* and *D*. Correspondence of membrane protein and actin distributions were analyzed with positive transparencies. (×869.)

of which spans the membrane (6, 13, 17). They are clearly integral proteins.

The cells we have studied were well-spread NRK and human fibroblasts in monolayer culture. Inside such flattened normal cells, filaments (stress fibers) are found in more-or-less parallel arrays often extending over much of the length of the cell. The stress fibers contain myosin (18), actin (19), and other mechanochemical proteins (20, 21). [For reasons of convenience, only actin distributions were examined in the present experiments, but the coincidence of actin and myosin in the stress fibers has been amply demonstrated in earlier studies (12, 18).] Most of these fibers are closely apposed to the upper or lower cytoplasmic surfaces of the cell, while others are located in the cell interior. Within any one cell, these can be distinguished by changing the microscope focus.

Our results indicate that initially the three different mem-

brane proteins were mobile in the plane of the membrane.‡ This is shown by the clustering that their respective antibodies induced, especially in ruffles and adjacent surface areas that were devoid of underlying stress fibers. This initial mobility, however, was apparently not equal in all directions in the plane of the membrane. These proteins appeared initially to be largely excluded from those regions of the membrane that were directly over stress fibers. This is suggested by the interdigitated (in projection) linear arrays of membrane proteins and stress fibers seen initially (Fig. 1 *A*, *B*, *E*, and *F*).

When the protein is cross-linked by its antibody reagents (a secondary antibody is required for β_2-microglobulin), however, the resulting clusters of each membrane protein became linked through the membrane to the stress fibers lying underneath. This is the only reasonable explanation of the superposition of the antibody-induced patches of the membrane proteins directly over the stress fibers (Fig. 1 *C*, *D*, *G*, and *H*). The possibility that this superposition is an artifact due somehow to Fc receptors in the fibroblast surface membrane is eliminated by the fact that the redistribution produced by $(Fab')_2$ fragments, lacking Fc, was indistinguishable from that produced by the whole antibodies. The formation of the transmembrane linkage is apparently energy-independent because it occurred even in the presence of 20 mM NaN_3.

This ligand-induced transmembrane linkage is the same result previously obtained upon clustering Con A receptor proteins by Fl-Con A on the surfaces of NRK cells (4). Since the three membrane proteins, aminopeptidase, β_2-microglobulin, and Na^+,K^+-ATPase, are apparently unrelated to one another and the Con A receptors probably constitute a large group of otherwise unrelated glycoproteins, the conclusion we draw is that quite generally *the clustering of integral membrane protein by an external multivalent ligand leads to an energy-independent linkage of such clusters to actin- and myosin-containing components inside the cell.* The fact that colchicine does not affect the linkage indicates that microtubules are not involved in the phenomenon.

The formation of such ligand-induced transmembrane linkages has been quite independently inferred from recent studies in our laboratory* (8) on the capping of membrane components on lymphocytes and other cells in suspension. It is well known (for review, see ref. 22) that when a multivalent ligand is bound to its specific receptors in the surfaces of many cells in suspension, such as lymphocytes, there often results first a clustering of the receptors into small patches over the surface in an energy-independent step, after which the patches are collected into a single cap over one area of the surface. We have shown, by double-fluorescent staining experiments, that accompanying this surface patching and capping of any one of a large number of different receptors there is always a concentration of intracellular actin and myosin components immediately under the patches and caps. We have proposed (8) that in general the mechanism of capping consists of two stages: (*i*) the ligand-induced clustering of a specific receptor leads to an energy-independent transmembrane linkage of that receptor cluster to actin and myosin components on the inside surface of the cell membrane; and (*ii*) such actin- and myosin-linked patches are then dragged into a cap by an analogue of the sliding filament mechanism of muscle (23). For our present purposes, the significant point is that for these two apparently different phenomena, namely, the immobilization of surface receptors on flattened fibroblasts and the capping of surface receptors on lymphocytes and other cells, the same early molecular events are implicated: a ligand-induced clustering of integral membrane proteins leading to a transmembrane linkage of the clusters to actin and myosin components underneath the membrane. The important difference seen between the fibroblasts and the lymphocytes, it is suggested, is the different states of the actin and myosin in the two kinds of cells. In the flattened normal fibroblast, the actin and myosin are organized in large part into stress fibers. Linkage of the membrane protein clusters to actin and myosin thus leads to the immobilization of the clusters. In the rounded lymphocyte and other cells in suspension, on the other hand, such stress fibers are not present, and the actin and myosin are probably not highly organized. The transmembrane linkage of clusters to actin and myosin in this type of cell forms a complex (patch) that is still mobile in the plane of the membrane, and such patches can be moved through the membrane and collected into a cap.

The formation of such ligand-induced transmembrane linkages is most probably the explanation for the immobilization of Con A receptors on myoblast cell surfaces observed by photobleaching experiments (24).

If the evidence is accepted that the formation of such transmembrane linkages is a general phenomenon applicable to many if not all membrane integral proteins, then what is the molecular mechanism for the linkages? This has been discussed to some extent in an earlier paper (8), and that discussion will only by summarized here. First, in all eukaryotic cells the actin appears to be in part associated with the plasma membrane and in part present in the interior cytoplasm (25). To account for the membrane binding of the ordinarily soluble actin, it is proposed that actin is a peripheral protein (1) bound directly or indirectly to a specific integral protein or proteins (X) of the plasma membrane. The myosin is presumed to be bound to the actin. It is further suggested that while X is thus attached to actin and myosin, it is not attached to any isolated, nonclustered membrane integral protein. However, when any such integral protein is clustered by its specific external ligand, the cluster then becomes bound to X and, hence, to actin and myosin. A possible analogue for this proposed interaction is the binding of the complement component C1q to aggregates of appropriate immunoglobulin molecules but not to their monomolecular forms (26).

There is no direct evidence for the putative protein(s) X. In principle, however, one way that it could be identified is by experiments with the cultured fibroblasts. X would be that unique integral protein whose specific fluorescent-labeled antibodies would be distributed *initially* (i.e., on fixed cells) in linear surface arrays that were *superimposable* on (rather than interdigitated with) the linear arrays of stress fibers under the membrane. By that criterion, the histocompatibility antigen is not X (Fig. 1*B*), a thought that we at one time entertained.

Evidence that is at least consistent, however, with the existence of such an integral protein X is provided in Fig. 1 *A*, *B*, *E*, and *F*. The exclusion *initially* of β_2-microglobulin, aminopeptidase, and the Na^+,K^+-ATPase from regions of the membrane directly above stress fibers may be the consequence of the predicted concentration of X in just those regions of the membrane (although other explanations for the exclusion are certainly possible). The completely uniform initial distribution of Fl-Con A on these same cell surfaces (4) would then be ex-

‡ The finely punctate fluorescence patterns seen on the surfaces of cells that were first fixed and then stained (Fig. 1 *B* and *F*) may indicate that a limited degree of clustering of the integral proteins is induced by their specific antibodies despite the fixation. In other words, the fixation used may not completely inhibit the short-range mobility of the membrane proteins.

plained if X, along with many other surface glycoproteins, were a Con A receptor.

In view of the apparent generality of this transmembrane linkage phenomenon, it may well play an important role in cell physiology, particularly in the transduction of external signals by cell membranes. Mitogens and antigens that stimulate appropriate cells to proliferate and differentiate are generally multivalent ligands for specific cell surface receptors and are often capable of clustering these receptors in the plane of the membrane. It has been proposed that hormones may also act to induce a clustering of their specific membrane receptors (27, 28). The clustering of their receptors by these physiologically relevant ligands may then induce the linkage of the specifically clustered receptors to intracellular actin and myosin that we have discussed above. The function of such a transmembrane linkage may be to provide the local molecular machinery to produce the specific endocytosis of the ligand-receptor patches (8); such endocytosis may quite generally be an essential step in the mechanism of action of these ligands. (For a specific example, see ref 29.)

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