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Identification of a protein kinase activity that phosphorylates connexin43 in a pH-dependent manner

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Abstract

The carboxyl-terminal (CT) domain of connexin43 (Cx43) has been implicated in both hormonal and pH-dependent gating of the gap junction channel. An *in vitro* assay was utilized to determine whether the acidification of cell extracts results in the activation of a protein kinase that can phosphorylate the CT domain. A glutathione S-transferase (GST)-fusion protein was bound to Sephadex beads and used as a target for protein kinase phosphorylation. A protein extract produced from sheep heart was allowed to bind to the fusion protein-coated beads. The bound proteins were washed and then incubated with ^{32}P-ATP. Phosphorylation was assessed after the proteins were resolved by SDS-PAGE. Incubation at pH 7.5 resulted in a minimal amount of phosphorylation while incubation at pH 6.5 resulted in significant phosphorylation reaction. Maximal activity was achieved when both the binding and kinase reactions were performed at pH 6.5. The protein kinase activity was stronger when the incubations were performed with manganese rather than magnesium. Mutants of Cx43 which lack the serines between amino acids 364-374 could not be phosphorylated in the *in vitro* kinase reaction, indicating that this is a likely target of this reaction. These results indicate that there is a protein kinase activity in cells that becomes more active at lower pH and can phosphorylate Cx43.

Key words
- Connexin
- Phosphorylation
- Phosphotransferases
- Protein kinase

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Introduction

Connexin43 (Cx43) is one of a group of integral membrane proteins that form intercellular channels called gap junctions. Six connexin proteins form a hexameric structure called a connexon, which constitutes a hemichannel. Two connexons, each provided by respective neighboring cells, can be aligned and assembled to form an intercellular channel.

All connexins share a common hydropathy profile consisting of four membrane spanning domains, one cytoplasmic loop (CL) and two extracellular loops (EL 1, EL 2); both amino and carboxyl terminals (AT and CT, respectively) seem to be located in the intracellular space. Interestingly, partial truncation of the CT has not prevented functional expression in most connexins tested, including: Cx32 (1), Cx37 (2), Cx40 (2), Cx43 (3), Cx45 (4), and Cx50 (5,6). The latter suggests

that the CT is not an essential component of the channel pore. It has been demonstrated that the CT domain of Cx43 plays an important role in regulation of intercellular communication. Changes in Cx43-mediated gap junctional communication by such factors as acidification, growth factors, oncogenes and activators of protein kinases have been shown to require an intact CT domain.

Cx43 is commonly found as a phosphoprotein. A number of potential consensus sites for both serine/threonine and tyrosine kinases can be identified. Detailed biochemical studies have shown that Cx43 acts as a substrate for phosphorylation by v-Src (7,8), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (9-11). Recent evidence also suggests that Cx43 was phosphorylated by the mitosis-associated cdc-2 kinase (12) and cGMP-dependent phosphorylation of rat Cx43 has also been demonstrated (13). The functional consequence of Cx43 phosphorylation is rather complex. For example, activation of PKC leads to a shift in the unitary conductance of the channel toward lower conductance states (14). However, there is a seemingly paradoxical increase in macroscopic conductance, probably due to an increase in open probability (15,16). These results show that electrical and metabolic coupling are not necessarily directly related, and can be regulated differently. Phosphorylation of Cx43 by MAPK also leads to a decrease in coupling (17,18). With respect to tyrosine kinases, co-expression of v-Src leads to phosphorylation of Cx43 and prevents the formation of junctional conductance in Cx43-expressing oocytes and this regulation has been shown to be dependent on the CT domain (19). Finally, it is interesting to note that the Src-homology 3 (SH3) domain of v-Src binds to the carboxyl terminal domain of Cx43 (7). The latter opens the possibility that the CT of Cx43 may associate with SH3-containing proteins thus modifying the degree of communication between cells.

Our long-term goal is to understand the mechanisms responsible for chemical regulation of gap junctions. We have focused on the molecular steps involved in the closure of Cx43 by low intracellular pH (pHi). Several protein kinases are activated under conditions of lower intracellular pH including MAPK (20), pp60 Src (21) and stress-activated protein kinases (SAPK) (22). Interestingly, both Src and MAPK are known to phosphorylate Cx43 in the CT domain. Due to the requirement for the CT domain for regulation by both lower pHi and protein kinases, we sought to determine whether we could detect a protein kinase activity that would result in the phosphorylation of Cx43 under low pH-dependent conditions. To do this we have generated a fusion protein consisting of glutathione-S-transferase (GST) with the CT domain of Cx43. We refer to this 40-kDa fusion protein as GST-Cx43-CT. We used an *in vitro* assay to determine whether lower pH could increase the binding to or phosphorylation of Cx43-CT by a protein kinase present in a cell or tissue extract. We were able to demonstrate that a protein kinase activity did exist that phosphorylated Cx43-CT on a serine residue and that this phosphorylation was greatly enhanced if the reaction occurred at low pH (6.5).

Experimental procedures

Production of Cx43 fusion proteins

The cytoplasmic tail domain of connexin 43 (Cx43-CT) was cloned, using the polymerase chain reaction, into the bacterial expression vector, pGEX-2T (Phamacia, Uppsala, Sweden). This vector produces fusion proteins of Cx43-CT covalently bound to GST. The template for the cloning of Cx43 was rat connexin 43 provided by Dr. David Paul (Harvard University). Mutant connexin 43 templates were produced as previously described (23). Deletion mutants are identified by the numbers of the deleted

amino acids. We studied the phosphorylation of the following Cx43 mutants; 261-280, 281-300 and 364-373.

To clone the wild type or mutant CT domains the following primers were used: forward GAA GGA TCC **ATG** AGC GAT CCT TAC CAC GCC and reverse - GCT TGA ATT CCA AGC CGG TTT AAA TCT CC. PCR was performed with *pfu* polymerase (Stratagene, La Jolla, CA, USA). After ligation into pGEX-2T, the plasmids were grown in *E. coli* DH5α. The fusion protein was expressed by incubating the bacteria in LB broth with 0.5 mM IPTG for 3-5 h. The bacteria were washed in Tris-buffered saline (TBS) and resuspended in TBS (1/10 of the volume of the LB broth culture) with 1 mM DTT. The cells were then sonicated on ice for 1 min. After sonication, NP-40 and PMSF were added to final concentrations of 1% and 1 mM, respectively. The extract was then cleared by centrifugation for 20 min at 17,000 *g* and the extracts were stored at -70°C. The presence of the fusion protein was confirmed by polyacrylamide gel electrophoresis (SDS-PAGE). To prepare fusion protein-coated beads, 1 ml of bacterial lysate was bound to 50 µl of glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 2.5 h while gently rocking. After loading, the beads were washed with TBS. The beads were then incubated with 2 mM ATP and 10 mM $MgCl_2$ for 20 min at 37°C in order to remove a high molecular weight contaminant from the beads. The beads were then washed three times with TBS and stored for up to one week in 1 ml of TBS with 1% NP-40 and 1 mM DTT.

Heart extract preparation and *in vitro* kinase assay

Sheep heart extracts were prepared by homogenizing 1 g of tissue (ventricle) in 10 ml of lysis buffer (50 mM MOPS (3-[N-morpholino] propanesulfonic acid, pH 7.5), 50 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.1% ß-mercaptoethanol, 1% Triton X-100, and complete protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). The extracts were cleared by centrifugation (14,500 rpm, 20 min), assessed for protein content by the method of Bradford, and stored at -70°C until use.

The *in vitro* kinase assay was derived from the assay system described by Turner et al. (24,25). For analysis of *in vitro* protein kinase activity, the fusion protein-coated beads were washed once and resuspended in 500 µl of lysis buffer. For each individual tube of the kinase assay, 30 µl of these beads were placed in 1 ml of lysis buffer (adjusted to the appropriate pH) with 1 mg (protein content) of sheep heart extract. Binding of the extract to the beads was at 4°C for 90 min with gentle rocking. Following binding, the beads were then washed three times with lysis buffer and then washed two times in kinase buffer (50 mM MOPS, 3 mM $MnCl_2$, again at the appropriate pH).

The pellet was resuspended in 20 µl of kinase buffer and 5 µCi of [γ-^{32}P] ATP (4500 Ci/mmol, ICN Biomedicals, Inc., Costa Mesa, CA, USA). Unless otherwise noted, the kinase reaction was for 20 min at 20°C. The reaction was terminated by the addition of 6X SDS sample buffer and the sample was placed in boiling water for 5 min. Samples were then resolved by SDS-PAGE, using an 11% resolving gel. Following SDS-PAGE, the proteins were visualized by staining with Coomassie blue (GelCode®, Pierce Chemical Co., Millford, IL, USA). Gels were dried, and analyzed by PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA).

Results

We utilized an *in vitro* kinase assay to determine whether cells had an activity that could result in the phosphorylation of connexin43. This assay utilized a construct of Cx43-CT fused to GST. Tissue extracts derived from sheep heart were prepared and

allowed to interact with the GST-CT fusion protein bound to a Sepharose bead. After binding, the beads were washed to remove unbound proteins and incubated with [γ-^{32}P] ATP. The proteins were resolved by SDS-PAGE and phosphorylation was assessed by PhosphoImager analysis. Figure 1 represents an example of one such experiment. When both the binding and kinase steps of this assay were performed at pH 7.5 there was minimal phosphorylation of the GST-CT fusion protein. Under these conditions, there was also minimal phosphorylation of a second protein of approximately 60 kDa. In previous studies, we have determined that an intracellular pH of 6.5 was sufficient to cause the closure of Cx43 channels expressed in *Xenopus* oocytes. When both the binding and kinase steps were performed at pH 6.5 there was a dramatic increase in the phosphorylation of GST-Cx43-CT and a significant increase in phosphorylation of the 60-kDa protein. No detectable phosphorylation of a control GST protein was observed (data not shown). When only the kinase or binding step was performed at pH 6.5 the result was an intermediate level of phosphorylation. This indicates that the kinase was both better retained in the bead-GST-CX43-CT complex at lower pH and was more active at lower pH.

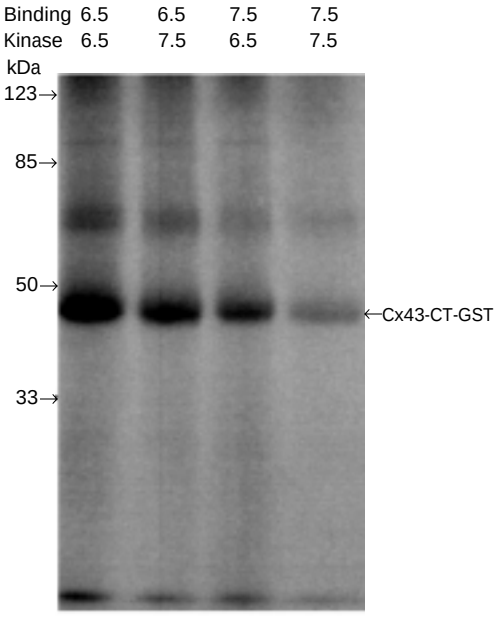

Figure 1 - Phosphorylation of Cx43. An *in vitro* kinase reaction was performed using a Cx43-CT-GST fusion protein. The fusion protein was immobilized on glutathione Sephadex resin and incubated with 1 mg of sheep heart extract for 90 min. Binding reactions were performed in MOPS buffer at either pH 6.5 or 7.5. After washing, the beads were incubated with 5 µCi of [γ-^{32}P] ATP in 50 mM MOPS and 3 mM $MnCl_2$ at the pH indicated. The reaction was stopped by the addition of SDS sample buffer and the proteins were resolved by SDS-PAGE. Visualization was performed using a PhosphoImager. The image shown was representative of at least three experiments. The migration of the Cx43-CT-GST fusion protein is marked. Phosphorylation is enhanced when the binding and/or kinase incubations are at pH 6.5. CT-GST, Carboxy terminal-glutathione-S-transferase fusion protein.

In order to determine whether this might be a general activation of protein kinases, we produced a second GST fusion protein construct with Cx46-CT. This protein was produced and used in a manner identical to Cx43. Both Cx43-CT and Cx46-CT, GST protein constructs, were used as targets for binding and phosphorylation by sheep heart extracts. Figure 2 shows the phosphorylation of Cx43-CT-GST fusion proteins at pH 6.5 and pH 7.5 (for both the binding and kinase steps). As expected, there was an increase in phosphorylation of Cx43-CT. When Cx46-CT was used in place of Cx43 there was no detectable phosphorylation at pH 7.5 and only a small, although detectable, phosphorylation at pH 6.5. The amount of connexin-GST fusion protein was shown to be similar based on the intensity of Coomassie staining. This phosphorylation reaction was, therefore, not a general increase in protein kinase activity but had some specificity. Testing of CT constructs derived from other connexin proteins would be required to determine the range of possible substrates of this protein kinase.

In order to better define the role of pH in the binding and kinase assays, the pH of one of these steps was held constant while the pH of the other was varied. In panel A of Figure 3 the kinase assay was held at either pH 6.5 (open bars) or pH 7.5 (closed bars), while the pH of the binding reaction was changed. The results were quantified and then normalized so that the highest value was 100%. As expected, the curve obtained when the kinase assay was performed at lower pH was significantly higher. This curve indicates that the lower the pH, the more kinase can bind to the GST-Cx43-CT. Due to the very low level of activity in this experiment, it is not possible to make a definitive comment about the kinase activity obtained when the kinase reaction was performed at pH 7.5. In panel B

of Figure 3, the pH of the binding step was held constant at either pH 6.5 (open bars) or pH 7.5 (closed bars). The pH of the kinase assay was then altered as indicated in the chart. The activity was higher when the binding was performed at the lower pH as is expected from the results shown in panel A. At both pHs of binding, the activity was higher when the kinase reaction was performed at a lower pH, reaching a maximum at pH 6.5. However, unlike the binding step, this activity did not seem to continue to increase as the pH was reduced.

In our initial studies, the kinase assays were performed in a buffer that utilized manganese as the source of divalent cations. In order to further characterize the kinase activity the experiments were performed with either 3 mM $MgCl_2$ or 3 mM $MnCl_2$. Figure 4 demonstrates that the activation of the protein kinase for phosphorylation of both the GST-Cx43-CT protein and the other phosphorylation targets in the extract was greatly enhanced by the presence of manganese rather than magnesium.

To further analyze the phosphorylation reaction, mutants of Cx43-CT were cloned into the GST expression vector. These proteins were expressed and analyzed for reaction with the acidification-activated protein kinase activity as before. The results of one such experiment are presented in Figure 5. When wild type Cx43-CT was used there was a significant increase in phosphorylation seen when the pH was dropped from 7.5 to 6.5. Conversely, no phosphorylation was observed when GST alone was used as a potential phosphate acceptor. Mutants of Cx43-CT were chosen based on the presence of potential phosphorylation sites. A deletion of the protein from amino acid 261 to 280 was phosphorylated in an acidification dependent manner although the overall level of phosphorylation was slightly less intense than that observed for the wild type protein. Similarly, there was phosphorylation of a mutant peptide that lacked amino acids 281 to 300. There was, however, a dramatic loss of demonstrable kinase activity when a deletion of amino acids 364-373 was tested. This deletion removes six serines that make up consensus sites for several protein kinases. From these results, the site

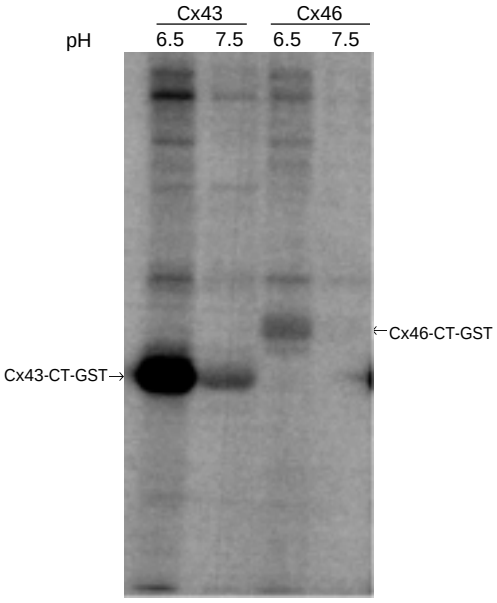

Figure 2 - Comparison of *in vitro* phosphorylation of Cx43 and Cx46. GST (glutathione-S-transferase) fusion proteins derived from Cx43 or Cx46 were prepared and analyzed for phosphorylation by sheep heart extracts. The pH of both the binding and kinase reactions was the same and is indicated in the figure. The results indicate that the kinase reaction was enhanced against Cx43 at lower pH.

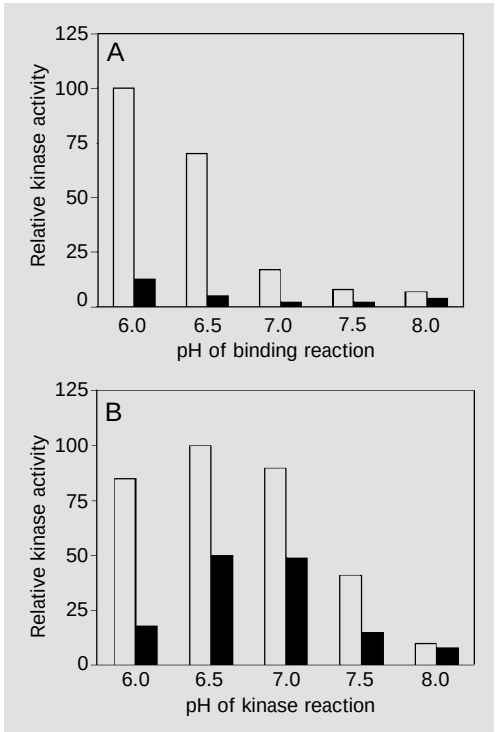

Figure 3 - Titration of pH in the *in vitro* kinase assay. In this assay, the pH of either the binding or kinase reaction was held constant while the pH of the other reaction was varied. The phosphorylation of the Cx43-CT-GST fusion protein was quantified by PhosphoImager analysis. Two lanes for each pH were quantified and the results were averaged. The figure is representative of three experiments. *Panel A*, The pH of the kinase reaction was held at either pH 6.5 (open bars) or pH 7.5 (closed bars) while the pH of the binding reaction was varied from 6 to 8 at 0.5-pH unit intervals. *Panel B*, The pH of the binding reaction was held at either pH 6.5 (open bars) or pH 7.5 (closed bars) while the pH of the kinase reaction was varied from 6 to 8 at 0.5-pH unit intervals.

of phosphorylation appears to be primarily in the serines at the C-terminal end of the molecule.

Discussion

In this report, we demonstrate that tissue extracts contain a protein kinase or kinases that can specifically interact with the carboxyl terminal domain of Cx43 and phosphorylate the protein. The unique property of these kinases is that the interaction with, and the phosphorylation of, connexin are enhanced by reducing the pH to 6.5. This coincides with the intracellular pH required to gate Cx43. The assay system chosen requires that the interaction of the protein kinase with the target protein be able to withstand multiple washing steps. Therefore, we can conclude that a relatively strong binding interaction must take place between the kinase and Cx43. The phosphorylation was somewhat specific. There was no detectable phosphorylation of the GST carrier protein and only weak phosphorylation of the Cx46-CT domain used as a control.

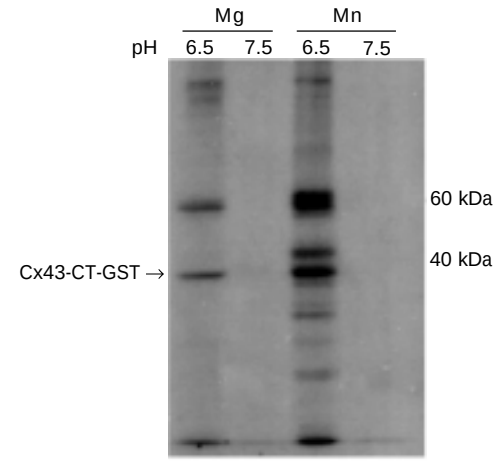

Figure 4 - The pH-dependent kinase is more active in the presence of Mn than Mg. The *in vitro* kinase assay was performed at pH 6.5 and 7.5 (the pH of the binding and kinase steps was constant). The divalent cation present in the kinase reaction was either 3 mM $MgCl_2$ or 3 mM $MnCl_2$ as indicated. Kinase activity was significantly enhanced by the presence of manganese. CT-GST, Carboxyl terminal-glutathione-S-transferase fusion protein.

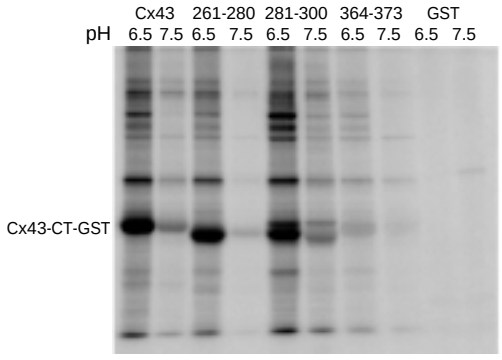

Figure 5 - Analysis of phosphorylation of Cx43 mutants. GST (glutathione-S-transferase) fusion proteins were prepared that had deletions in the CT (carboxyl terminal) domain of Cx43. The mutants tested had deletions from amino acids 261-280, 281-300 and 364-373. In addition, both wild type Cx43-CT and GST alone were tested. The mutants were tested with the reactions performed at both pH 6.5 and pH 7.5. The presence of equal amounts of fusion protein in the reaction was confirmed by the Coomassie blue staining of the gel. The migration of the Cx43-CT-GST fusion protein is marked. The mutated isoforms migrate slightly faster. The phosphorylation of the fusion protein was greatly reduced by the removal of the region from amino acids 364 and 373.

Many studies have shown that phosphorylation is a common modification of the connexin molecule (26, see also 27). Others have demonstrated that phosphorylation of Cx43 results in an alteration of the unitary conductance of connexin channels (14,28-30). The activation of kinases coincides with a shift of the unitary conductance of gap junction channels from neonatal cardiac myocytes (31). While under dephosphorylating conditions, human Cx43 channels show a predominance of larger unitary conductance (28). Furthermore, it has been suggested that permeability and single channel conductance of Cx43 gap junction channels are independently regulated, and that electrical and metabolic coupling are differentially modulated by various phosphorylating conditions (15). Taken together, these data suggest a relationship between phosphorylation of connexins and modification of intercellular communication.

We have previously demonstrated that the CT domain of Cx43 is required for acidification-induced gating (32,33). This domain is also required for other gating reactions (19,34). A number of protein kinases are known to phosphorylate Cx43 and modify the gating of the channels. In many cases, the phosphorylation reactions have been shown to occur in the CT domain. Truncation or mutation of the CT domain of Cx43 results in a loss of the ability of Cx43 to be modulated by a number of protein kinases or factors known to activate protein kinases. In one example, Src kinase was unable to induce the gating of a truncated form of Cx43 (19) unless the CT domain was also expressed in the cell. In another example, mu-

tation of three serines that represent potential MAPK phosphorylation sites reduces the ability of growth factors to gate connexins (17). It is possible that the acidification-induced phosphorylation of connexin described in this manuscript may act as a mediator in acidification-induced gating.

It has been suggested that many regulators of cell-to-cell coupling act in concert. For example, pHi and Ca have been reported to act together to regulate channel activity (35-37). More recent studies have proposed that acidification-induced gating does not directly affect cell coupling, but may act through the ubiquitous calcium-receptor protein, calmodulin (38). It has also been suggested that calcium-induced gating of gap junction channels could result from the activation of specific kinases, with consequent connexin phosphorylation (39). It is interesting to note that there are multiple calcium/calmodulin-dependent kinase II (CAMK II) phosphorylation sites in the carboxyl-terminal domain of Cx43.

Our studies localized the region of phosphorylation to a serine-rich region at the C-terminal end of the Cx43 molecule. This region contains consensus phosphorylation sites for PKC, cAMP-dependent protein kinase and CAMK. To date, our studies have not been able to specifically detect any of these kinases in the proteins bound to Cx43-CT at lower pH. We have also not been able to block the acidification-induced phosphorylation reactions using inhibitors of these kinases. Future studies will be performed to identify the kinase or kinases involved.

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