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Adhesion-Dependent Degranulation of Neutrophils Requires the Src Family Kinases Fgr and Hck

Attila Mócsai,1,2,5 Erzsébet Ligeti,1 Clifford A. Lowell,2 and Giorgio Berton3,5

Polymorphonuclear neutrophils (PMN) adherent to integrin ligands respond to inflammatory mediators by reorganizing their cytoskeleton and releasing reactive oxygen intermediates. As Src family tyrosine kinases are implicated in these responses, we investigated their possible role in regulating degranulation. Human PMN incubated on fibrinogen released lactoferrin in response to TNF-α and this response was inhibited by PP1, a Src family tyrosine kinase inhibitor. This drug had no effect on lactoferrin secretion induced by PMA, an adhesion-independent agonist of PMN degranulation. However, PP1 blocked secretion in PMN plated on plain tissue culture plastic, a surface inducing PMN spreading in the absence of any stimulus. Double knockout hck−/−fgr−/− PMN adherent to collagen or fibrinogen failed to release lactoferrin in response to TNF-α but responded to PMA as wild-type PMN. Degranulation induced by spreading over tissue culture plastic was also defective in hck−/−fgr−/− PMN. Defective adhesion-dependent degranulation required the absence of both kinases, because single knockout fgr−/− or hck−/− PMN responded as wild-type cells. Analysis of lactoferrin secretion in hck−/−fgr−/− or PP1-treated, treated PMN showed that Src kinases are not implicated in degranulation dependent on activation of protein kinase C or increase in intracellular free Ca2+ but may play a role in the response to FMLP of cytochalasin B-treated PMN. These findings identify a role for Src family kinases in a signaling pathway leading to granule-plasma membrane fusion and suggest that Fgr and Hck would be targets for pharmacological control of adhesion-dependent degranulation in the inflammatory site.


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4 Abbreviations used in this paper: PMN, polymorphonuclear neutrophils; ROI, reactive oxygen intermediates; TNF, TNF-α; PKC, protein kinase C; CB, cytochalasin B.

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Polymorphonuclear neutrophils (PMN)4 adherent to extracellular matrix proteins or endothelial counterreceptors respond to inflammatory mediators, such as cytokines and chemoattractants, releasing reactive oxygen intermediates (ROI) and granule constituents (1–5). These secretory responses are tightly coupled with a rearrangement of the cytoskeleton that dictates spreading of the cell over the adhesive surface (5–8). Different adhesion receptors have been implicated in induction of PMN responses. For example, both β2 and β1 integrins have been demonstrated to regulate PMN responses (2, 3, 6, 9–11). The release of ROI and degranulation in the context of PMN-endothelial cell interactions plays a central role in the development of inflammatory reactions and certainly contributes to tissue damage associated with inflammation (12, 13).

In the last few years, a great progress has been made in the understanding of signaling from adhesion receptors (14, 15). Adhesion and spreading of PMN are tightly coupled to an increase in tyrosine phosphorylation of several proteins, and tyrosine kinase inhibitors block adhesion-dependent generation of ROI by PMN (7, 8). The Src family tyrosine kinases Fgr, Hck, and Lyn, as well as p72syk, have been recently implicated in signaling from adhesion receptors (16–19).

Signals involved in triggering degranulation by adherent PMN are poorly understood. The evidence that protein tyrosine phosphorylation (7, 16) and the Src family kinases Fgr and Hck (18) play a critical role in ROI generation prompted us to investigate whether adhesion-dependent degranulation is also regulated by the same signaling pathways. In this report, we show that adhesion-dependent release of lactoferrin is blocked by a newly described tyrosine kinase inhibitor the effect of which has been reported to be selective for Src family tyrosine kinases (20). Investigations with PMN isolated from mice with the double deficiency of Fgr and Hck allowed us to demonstrate that these kinases play an essential role in signaling for adhesion-dependent degranulation. These results provide the first direct evidence that Src family kinases function in a signaling pathway leading to granule-plasma membrane fusion.

Materials and Methods

PMN isolation

Human PMN were isolated from Buffy coats of healthy volunteers as previously described (9, 16). Wild-type (C57BL/6), p59/61c-fgr, or p58/68fgr, deficient single knockout mice and double knockout mice deficient in both p59/61c-fgr and p58/68fgr were described previously (21). Bone marrow of 6–12-wk-old mice of both sexes were used in this study. Marine PMN were isolated essentially as described (18) except that a simplified three-layer gradient was utilized to separate neutrophils from other bone marrow cells (i.e., bone marrow cells suspended in Ca2+/Mg2+-free HBSS supplemented with 0.1% BSA were layered on the top of a 62/81% two-layer Percoll (Pharmacia, Uppsala, Sweden) gradient, and after centrifugation, PMN were harvested from the 62/81% interface). At the end of the preparation, PMN were suspended in ice-cold HBSS containing 0.5 mM CaCl2, 1 mM MgCl2, and 5 mM d-glucose (HBSS) and kept in ice until use. For experiments in which the Mg2+ dependence of the PMN response was
investigated, cells were isolated as above described but resuspended in Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS supplemented with 5 mM EDTA and, after 10 min of incubation, washed and suspended in HBSS either with or without 1 mM Mg\(^{2+}\) (17).

**Coating of tissue culture plastic with proteins**

Flat bottom polystyrene tissue culture plates with 96 wells (Greiner, Frick- enhausen, Germany) were left untreated or were covered with rat collagen, human fibrinogen, or FCS as described (18). After coating, the plates were washed once with PBS and once with HBSS.

**Cell stimulation**

Human and murine PMN were washed at 4°C and resuspended in ice-cold HBSS at 1 x 10\(^6\) or 2 x 10\(^5\) cells/ml, respectively. For adhesion assays, cell suspensions (100 μl/well), added or not with 10 μM PP1 (Calbiochem-Novabiochem Int., La Jolla, CA) were dispensed in protein-coated plates and prewarmed for 10 min at 37°C before addition of 20 ng/ml human or murine TNF-α (TNF, Peprotech, London, U.K.), 100 ng/ml PMA (Sigma, St. Louis, MO), or diluent alone. Since the TNF stock solution was stored in the presence of BSA, all the other samples were supplemented with the same concentration of BSA. After incubation at 37°C for the indicated times, samples were transferred to ice-cold polypropylene tubes and centrifuged at 8000 x g for 1 min at 4°C. Supernatants were used for the determination of granule marker release into the extracellular space. For suspension assays, 100-μl aliquots of a 2 x 10^6/ml cell suspension were dispensed in polypropylene tubes and either preincubated or not with 10 μM cytochalasin B (CB) and/or PP1 for 10 min at 37°C. PMN were stimulated with 1 μM FMLP (Sigma), 1 μM ionomycin, or 100 ng/ml PMA, and after 10 min samples were transferred to ice-cold polypropylene tubes and processed as described above for adhesion assays.

**Determination of lactoferrin release**

Release of the specific granule marker lactoferrin was measured by an ELISA using anti-human lactoferrin Abs (Sigma). Supernatants of human or murine PMN were diluted 4- or 20-fold, respectively, in 50 mM CO\(_2\)-free HCO\(_3\)-buffer (pH 9.6). A total of 100 μl of the diluted supernatants or of known concentrations of human lactoferrin were added to Nunc Maxisorp F96 (Nunc, Roskilde, Denmark) immunoplate wells and incubated overnight at 4°C. All subsequent steps were conducted at room temperature and separated by several washings. Non-specific binding sites were blocked with PBS supplemented with 0.5% BSA and 0.5% Tween 20 (blocking solution). Plates were then incubated with affinity-purified rabbit anti-human lactoferrin (Sigma; dilution, 1/500) followed by peroxidase-conjugated anti-rabbit Ab (Amersham, Little Chalfont, U.K.; dilution, 1/5000), both dissolved in blocking solution. Color was developed by the o-phenylenediamine/H\(_2\)O\(_2\) system. Absorbance of the wells were read at 490 nm with a Packard Arcus 300 microplate reader, and the lactoferrin concentration was calculated using the human lactoferrin calibration curve. With both human and murine samples, no signal was detected if the anti-lactoferrin Ab was substituted with an irrelevant rabbit antisera. The reactivity of the anti-human lactoferrin Ab with murine lactoferrin was determined by Western blotting. Murine PMN suspended at 10^6/ml in HBSS were incubated for 15 min at 37°C in the presence or the absence of 100 ng/ml PMA. Cells were then spun down and the supernatant solubilized in 4X concentrated SDS-PAGE sample buffer and boiled for 10 min. Samples were run on a 10% SDS-PAGE, electrophoreted onto nitrocellulose sheets (Hybond C, Amersham, Little Chalfont, U.K.; dilution, 1/5000), both dissolved in blocking solution. Chemiluminescence reagent. The anti-human lactoferrin Ab recognized one single band at ~78 kDa in supernatants of murine PMN (not shown). The m.w. of this band and the fact that its intensity increased in the supernatants of PMA-stimulated PMN confirmed this protein to be lactoferrin.

**Measurement of β-glucuronidase release**

Release of the primary granule marker β-glucuronidase from the same supernatants used for lactoferrin measurements was conducted essentially as described.

**Expression of the data**

All the experiments were performed in duplicates or triplicates. Since data obtained with mouse samples could not be exactly quantified using standard curves done with human lactoferrin, lactoferrin release from murine PMN is expressed as fold increase compared with the amount detected in supernatants of unstimulated PMN (see figure legends).

**Results**

**TNF-stimulated lactoferrin release by adherent human and murine PMN is blocked by the tyrosine kinase inhibitor PP1**

Human adherent PMN can be stimulated to mobilize lactoferrin-containing specific granules in response to granulocyte-macrophage-CSF, FMLP, or Ca\(^{2+}\) ionophore A23187 (5). As shown in Fig. 1A, TNF is also an effective agonist of lactoferrin secretion in adherent human PMN. As previously reported with other stimuli (5), TNF-induced degranulation by adherent PMN is delayed in its onset and prolonged up to 60 min of incubation (Fig. 1A). In accord with previous studies (4, 23–25), we could not detect release of the primary granule marker β-glucuronidase in response to TNF (data not shown). Additionally, we also found that the response to TNF depended on adhesion (25, 26), since suspended PMN failed to release lactoferrin following TNF stimulation while such cells did respond to PMA or ionomycin (data not shown; see Fig. 6 for data with murine PMN).

As a first approach to elucidate signals involved in degranulation by adherent PMN, we examined whether the compound PP1, which was recently described as a relatively selective inhibitor of Src family tyrosine kinases (20), affected TNF-stimulated lactoferrin secretion. As shown in Fig. 1A, 10 μM PP1 completely inhibited lactoferrin secretion in response to TNF. Importantly, PP1 inhibited only the adhesion-dependent response; in fact, PMA-induced degranulation was unaffected by the drug (Fig. 1B).

Previous evidence established a strict link between adhesion-dependent stimulation of PMN functions and cell spreading (5–8). In line with this evidence, we observed that PP1 completely blocked PMN spreading on fibrinogen in response to TNF, but not PMA (Fig. 2). In addition, generation of superoxide anion, which is also stimulated as PMN are induced to spread by TNF, was blocked by PP1 (L. Fumagalli and G. Berton, unpublished observation). It is unlikely that PP1 acts by inhibiting TNF receptor signaling because it also blocked lactoferrin secretion that occurred when PMN were plated on plain tissue culture plastic, a surface that can rapidly induce PMN spreading, in the absence of any stimulus (Fig. 1C).

The described inhibitory effects of PP1 on adhesion-dependent degranulation were also observed using PMN isolated from mouse bone marrow (Fig. 1D). As seen with human cells, murine PMN released lactoferrin when incubated on collagen in the presence of TNF or on plain tissue culture plastic in the absence of any stimulus (see also Fig. 3). PP1 inhibited murine PMN lactoferrin secretion in both conditions (Fig. 1D). The high degranulation response induced by PMA in mouse PMN was unaffected by PP1 (see legend to Fig. 1D). As found with human PMN (Fig. 2), PP1, in parallel with its inhibition of degranulation also completely blocked murine PMN spreading in response to TNF, but not PMA (data not shown).

**Adhesion-dependent lactoferrin release is defective in double knockout hck\(^{-/-}\)/fgr\(^{-/-}\) PMN**

Previous studies demonstrated that adhesion-dependent generation of superoxide anion is defective in hck\(^{-/-}\)/fgr\(^{-/-}\) PMN (18). Having established that adherence and spreading induce lactoferrin release in murine PMN and that the Src family selective inhibitor PP1 blocks this response in both human and murine PMN (Fig. 1), we addressed whether deficiency of Fgr and Hck results in a defective adhesion-dependent degranulation. Wild-type PMN released lactoferrin in response to TNF if incubated on collagen or, albeit to a lower extent, on fibrinogen, while they responded poorly if incubated on FCS (Fig. 3). In addition, wild-type PMN released lactoferrin when incubated on plain tissue culture plastic even in...
the absence of TNF. Comparable results were previously obtained by assaying superoxide anion release by wild-type PMN (18). Lactoferrin release in response to TNF by \( \text{hck}^{2/2} \text{fgr}^{2/2} \) double knockout PMN was defective on all the surfaces tested. Importantly, the spontaneous lactoferrin release by PMN incubated on tissue culture plastic was also totally defective in \( \text{hck}^{2/2} \text{fgr}^{2/2} \) PMN.

As lactoferrin release in response to TNF was optimal when PMN were adherent to collagen we investigated degranulation by collagen-adherent PMN in more detail. As shown in Fig. 4A, lactoferrin release by wild-type PMN adherent to collagen and stimulated with TNF was delayed and maximal at about 30 min. We did not detect any release of the primary granule marker \( \beta \)-glucuronidase under these conditions. \( \text{hck}^{2/2} \text{fgr}^{2/2} \) double knockout PMN did not release lactoferrin in response to TNF at any of the time points tested. However, PMA induced release of lactoferrin to a comparable extent in wild-type and double knockout PMN (Fig. 4B). Light microscopy observation confirmed previous findings (18) that \( \text{hck}^{2/2} \text{fgr}^{2/2} \) PMN did not spread in response to TNF but responded to PMA as well as wild-type cells.

Because adhesion-dependent production of superoxide anion is normal in single knockout \( \text{fgr}^{2/2} \) or \( \text{hck}^{2/2} \) PMN (18), we sought to investigate whether the knockout of the two kinases is required to cause a total deficiency of adhesion-dependent lactoferrin release. As shown in Fig. 5, double knockout, but not single knockout \( \text{fgr}^{2/2} \) or \( \text{hck}^{2/2} \), PMN were totally defective in their capability to release lactoferrin in response to TNF when plated on collagen. In addition, degranulation induced by spreading on tissue culture plastic was comparable in wild-type and single knockout PMN, but totally defective in the double knockout cells.

**Adhesion-independent lactoferrin release occurs normally in double knockout \( \text{hck}^{2/2} \text{fgr}^{2/2} \) PMN**

The findings described in Figs. 3–5 implicate Fgr and Hck in signaling for adhesion-dependent degranulation. However, the defective response of \( \text{hck}^{2/2} \text{fgr}^{2/2} \) PMN could be also due to the fact that Hck and Fgr play a more general role in signaling for degranulation and/or granule-plasma membrane fusion. As a first approach to demonstrate that Hck and Fgr are required for signaling from integrins, we addressed whether TNF-induced lactoferrin release by murine PMN is indeed dependent on adhesion. To this purpose, we exploited previous findings demonstrating that PMN adhesion in response to TNF is absolutely dependent on the presence of Mg\(^{2+}\) in the incubation medium (17). As shown in Fig. 6, in experiments in which lactoferrin release by murine PMN was investigated in suspension assays, TNF was unable to induce a response whether Mg\(^{2+}\) was included or not in the assay medium. However, wild-type, but not \( \text{hck}^{2/2} \text{fgr}^{2/2} \) PMN, released lactoferrin in response to TNF if incubated on collagen, and this response was strictly dependent on the presence of Mg\(^{2+}\). Since Mg\(^{2+}\) is required for recognition of extracellular matrix proteins by integrins, the data demonstrate that impairment of integrin-mediated adhesion in wild-type cells produced the same defect in degranulation seen in mutant PMN under normal culture conditions. This supports the model that the defect we found in
hck<sup>−/−</sup>fgr<sup>−/−</sup> PMN concerns an adhesion-dependent cell response.

To test directly whether hck<sup>−/−</sup>fgr<sup>−/−</sup> PMN were able to release lactoferrin in assays not requiring adhesion, we analyzed degranulation occurring in suspended PMN in response to different stimuli. As shown in Fig. 7A, lactoferrin release in standard suspension assays in response to PMA or the Ca<sup>2+</sup> ionophore ionomycin is comparable in wild-type and hck<sup>−/−</sup>fgr<sup>−/−</sup> PMN. Hence, Hck and Fgr do not regulate granule-plasma membrane fusion and are dispensable for lactoferrin secretion dependent on protein kinase C (PKC) and Ca<sup>2+</sup>. However, we found that hck<sup>−/−</sup>fgr<sup>−/−</sup> PMN were indeed defective in the response to FMLP (Fig. 7B). This finding suggests a role of Src family kinases in signaling by FMLP and is concordant with the evidence that FMLP activates Src family kinases in PMN (Refs. 27–29, see Discussion). It must be, however, noted that FMLP-induced lactoferrin secretion required disruption of the actin-based cytoskeleton with CB, while PMA- and ionomycin-induced degranulation occurred also in the absence of this drug (Fig. 7). At present, we do not know whether Hck and Fgr are implicated in signaling by FMLP and/or in facilitation of FMLP signaling by cytoskeletal rearrangements induced by CB.

To reinforce the evidence that Hck and Fgr are not implicated in PKC- and Ca<sup>2+</sup>-dependent lactoferrin secretion, but play a role in FMLP and CB-induced degranulation, we addressed whether the Src kinase inhibitor PP1 affects degranulation in suspension assays. As shown in Fig. 8A, PP1 inhibited the FMLP + CB-induced degranulation, albeit at a higher concentration than that required to block adhesion-dependent degranulation (see Fig. 1). However, lactoferrin release induced by PMA or ionomycin was unaffected by PP1 (Fig. 8B). We conclude that Src family kinase inhibition by PP1 affects PMN degranulation in the same fashion as the knock-out of hck and fgr, i.e., inhibits the response to FMLP + CB but does not modify the PKC- and Ca<sup>2+</sup>-induced response.

**Discussion**

Signaling from adhesion molecules in PMN involves Src family tyrosine kinases. In this report, we demonstrate that adhesion-dependent release of lactoferrin-containing specific granules is dependent on the activity of tyrosine kinases and on the expression of at least two members of the Src family, i.e., the Fgr and Hck proteins. PP1, which has been described as a selective inhibitor of Src family kinases (20), inhibits adhesion-dependent lactoferrin...
release from both human and murine PMN. In the absence of Fgr and Hck, PMN failed to mobilize specific granules after adhesion to different surfaces. PP1 does not affect specific granule exocytosis in response to either PMA or a Ca\(^{2+}\) ionophore. In addition, hck\(^{-/-}\)/fgr\(^{-/-}\) PMN responded normally to PMA and a Ca\(^{2+}\) ionophore. These findings strongly argue that Fgr and Hck are critically involved in transducing signals through adhesion molecules, but degranulation can still be induced by activating PKC with phorbol esters or increasing intracellular free Ca\(^{2+}\) concentrations. Hence, although Fgr and Hck were found to localize at least in part in PMN-specific and primary granules, respectively (30, 31), they are dispensable for PKC- and Ca\(^{2+}\)-induced degranulation.

As shown for ROI generation (18), adhesion-dependent degranulation requires cell stimulation with a cytokine, such as TNF (Refs. 4 and 23–26, and this article), or other agonists (4, 5). It is unlikely that Fgr and Hck are primarily involved in agonist signaling. In fact, stimulus-independent degranulation induced by spreading of PMN on tissue culture plastic was blocked by PP1 and was also defective in hck\(^{-/-}\)/fgr\(^{-/-}\) PMN. Additionally, TNF-induced up-regulation of CD11b/c is normal in hck\(^{-/-}\)/fgr\(^{-/-}\) PMN (18). The evidence that PP1 blocks human and murine PMN spreading and that hck\(^{-/-}\)/fgr\(^{-/-}\) fail to spread (18) suggests a strict link between PMN spreading and degranulation. Thus, it is most likely that signaling from adhesion receptors requires Src family tyrosine kinases to initiate cytoskeletal rearrangements leading to cell spreading and subsequent degranulation.

Analyzing degranulation in suspension assays, we found that hck\(^{-/-}\)/fgr\(^{-/-}\) PMN and PMN treated with the Src kinase inhibitor PP1 are defective in the response induced by FMLP in the presence of CB. These findings suggest that signaling by the FMLP receptor also involves Src family kinases. Indeed, several reports have implicated Src family kinases in signaling by FMLP in PMN (27–29), as well as by heterotrimeric GTP-binding protein-coupled receptors in other cell types (reviewed in Ref. 32). The data reported in Fig. 7 do suggest that Hck and Fgr play a critical role in FMLP signaling in PMN. Indeed, we have also found that activation of the respiratory burst by FMLP in CB-treated hck\(^{-/-}\)/fgr\(^{-/-}\) PMN is defective and that FMLP activates the Src family kinases Hck and Fgr in CB-treated human PMN (L. Fumagalli, C. A. Lowell, and G. Berton, manuscript in preparation). Activation of PMN responses by FMLP in murine PMN is absolutely dependent on the disassembly of the actin-based cytoskeleton by cytochalasins. In a previous study (18) and the present one, we invariably found that
hck^{−/−}fgr^{−/−} PMN display alterations in the organization of the actin cytoskeleton, which may contribute to their inability to spread over integrin ligands or even plain tissue culture plastic. Hence we cannot exclude that impairment of the response to FMLP results from cytoskeletal alterations present in double mutant PMN.

A clear hierarchy of mobilization of different granule compartments, i.e., secretory vesicles > gelatinase granules > specific granule (lactoferrin-containing) > azurophil granules, has been demonstrated in human PMN (33). To our knowledge, it is not known whether specific granules of murine PMN can be divided into gelatinase-containing and lactoferrin-containing subsets. Since hck^{−/−}fgr^{−/−} PMN are not defective in the capability to up-regulate CD11b/CD11c (18), it is tempting to speculate that Fgr- and Hck-regulated adhesion selectively controls mobilization of distinct granule subsets.

Although PMN spreading can occur independently of degranulation (5), previous studies (5) and those described in this report suggest that degranulation by adherent PMN requires cell spreading. PP1 blocked spreading of both human and murine PMN in response to TNF or FMLP, but not PMA, and hck^{−/−}fgr^{−/−} PMN were unable to spread on different surfaces (18). As with degranulation, ROI generation was also shown to be strictly correlated with spreading (18). Thus, it appears that loss of Src family kinase activity, either through the use of PP1 or in the knockouts, primarily affects PMN spreading which is in turn required for ROI generation and degranulation.

To some extent, it is surprising that degranulation proceeds in coincidence with the extensive process of actin polymerization that accompanies cell spreading. In fact, the block of actin polymerization by cytochalasins has been used since the early 1970s to enhance PMN degranulation (see Ref. 34 for a review of early studies). Additionally, at least with murine PMN, we could not detect any significant release of lactoferrin in FMLP-stimulated cells assayed in suspension if cells were not pretreated with CB (Fig. 7). Cytoskeleton rearrangement is a highly dynamic process (35). Thus, localized rearrangements of the actin cytoskeleton may provide a driving force for membrane fusion between adjacent granules and plasma membrane. In the absence of Hck and Fgr activity, the signals to initiate the appropriate cytoskeletal changes do not occur. Interestingly, members of the Rho subfamily of GTP-binding proteins that play a central role in actin polymerization have been described to regulate degranulation in mast cells (36–38).

PMN degranulation is thought to play an important role in tissue damage and development of inflammation (12, 13, 33). Importantly, we recently found that hck^{−/−}fgr^{−/−} mice are markedly...
resistant to the lethal effect of high dose lipopolysaccharide injection, and this correlates with reduction in liver and renal damage (39). Reduced production of reactive oxygen intermediate (18) and release of granule constituents (this article) by PMN adherent to the vascular wall of inflamed tissues may explain resistance of hck<sup>−/−</sup> fgr<sup>−/−</sup> mice to endotoxic shock. Implication of the Src family tyrosine kinases Fgr and Hck as regulators of adhesion-dependent PMN degranulation suggests that they would be new targets for pharmacological control of the inflammatory process.

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