# Secretion requires a cytoplasmically disposed sulphydryl of the RER membrane

**Robert C. Jackson*, Peter Walter & Günter Blobel**

Laboratory of Cell Biology, The Rockefeller University, New York, New York 10021

**The capacity of the rough endoplasmic reticulum (RER) membrane of eukaryotic cells to translocate nascent presecretory proteins from the cytosol to the intracisternal space is preserved on cell fractionation and can be assayed *in vitro*[1]. Two attempts to characterize this translocation activity have been reported. Warren and Dobberstein[2] reported that microsomal membranes can be depleted of their translocation activity by extraction with a solution of high ionic strength (500 mM KCl) and that activity can be restored to the depleted membranes by re-addition of the salt extract. On the other hand, Walter *et al.*[3] reported that KCl extraction of the microsomal membrane does not result in complete depletion of its translocation activity. However, mild trypsinization of the microsomal membrane released a tryptic fragment(s) from the membrane which, when recombined with a tryptically inactivated membrane fraction, restored translocation activity[3]. We now show that both the trypsin and the KCl extracted factors, but not the membrane-integrated remainder of the translocation apparatus, contain at least one sulphydryl group that is essential for activity.**

Our assay for the translocation activity of microsomal membranes takes advantage of the obligate coupling of translocation to proteolytic processing of nascent presecretory proteins[3-5]; hence, the ratio of 'processed' prolactin to 'unprocessed' preprolactin is a readily quantifiable measure of the translocation activity of the microsomal membrane.

It was previously noted[6] that *N*-ethylmaleimide, a sulphydryl-modifying reagent, inhibited translocation. Our data here confirm this observation. Preincubation of ribosome-stripped microsomal vesicles with 1.5 mM *N*-ethylmaleimide completely inhibited translocation, as virtually no processed prolactin was synthesized (Figs 1, 2, compare lanes 2 and 3).

A quantitative analysis of this inhibition by various concentrations of *N*-ethylmaleimide and other sulphydryl-modifying reagents (TLCK, a trypsin inhibitor, is mildly reactive with sulphydryl groups[7]) is shown in Fig. 3. We also analysed duplicate aliquots of these same modified samples by the signal peptidase-independent translocation assay described by Warren and Dobberstein[2]. In this, translocation activity is determined from the ability of the microsomal membrane to protect translocated polypeptides from proteolytic digestion, as quantitated by an increase in trichloracetic acid-precipitable radioactivity. Values comparable to those presented in Fig. 3 (data not shown) were obtained. *N*-Ethylmaleimide was the most effective inhibitor of translocation. At a concentration of less than 1.0 mM, *N*-ethylmaleimide completely inhibited translocation (Fig. 3*a*, *b*). Although the other sulphydryl-modifying reagents tested were also effective, concentrations in excess of 1.0 mM were required to achieve complete inhibition of translocation in the given conditions. Interestingly, the bound ribosomes of 'native' rough microsomes do not protect the sensitive sulphydryl group(s) from *N*-ethylmaleimide modification. As shown in Fig. 3*b*, *N*-ethylmaleimide-modified rough microsomes, when stripped of ribosomes by EDTA extraction and subsequently assayed for translocation activity, are inhibited to the same extent as an equivalent amount of microsomes which had been stripped of ribsomes before *N*-ethylmaleimide modification.

To rule out the possibility that the observed inhibition of the synthesis of processed prolactin could have resulted from an inactivation of signal peptidase rather than an impairment of translocation, *N*-ethylmaleimide-modified stripped microsomes were dissociated with sodium deoxycholate and assayed for signal peptidase activity in a post-translational assay[8,9]. The results (Fig. 4) demonstrate that signal peptidase is not inactivated by *N*-ethylmaleimide modification.

The fact that the translocation apparatus can be separated into soluble and membrane-associated fragments enabled us to localize the sensitive sulphydryl group(s) to either of these fragments. Soluble fragments were prepared as previously described[3], by subjecting EDTA-stripped and KCl-extracted microsomal vesicles to low concentrations of trypsin (7 $\mu g\ ml^{-1}$). Although such a mild treatment does not completely separate the membrane from the soluble portion of the translocation apparatus, it avoids the rapid inactivation of the solubilized fragments observed at higher concentrations of trypsin[3]. Centrifugation yielded a supernatant fraction that was further incubated in the absence or presence of *N*-ethylmaleimide. These fractions are referred to as $t_7$-sup or $t_7$n-sup, respectively.

Membrane-associated fragments, on the other hand, were prepared by digestion of EDTA-stripped, salt-extracted microsomal vesicles with a high concentration of trypsin (60 $\mu g\ ml^{-1}$). This harsher treatment generates a membrane fraction that is completely translocation inactive by itself (see Figs 1, 2, lane 7)

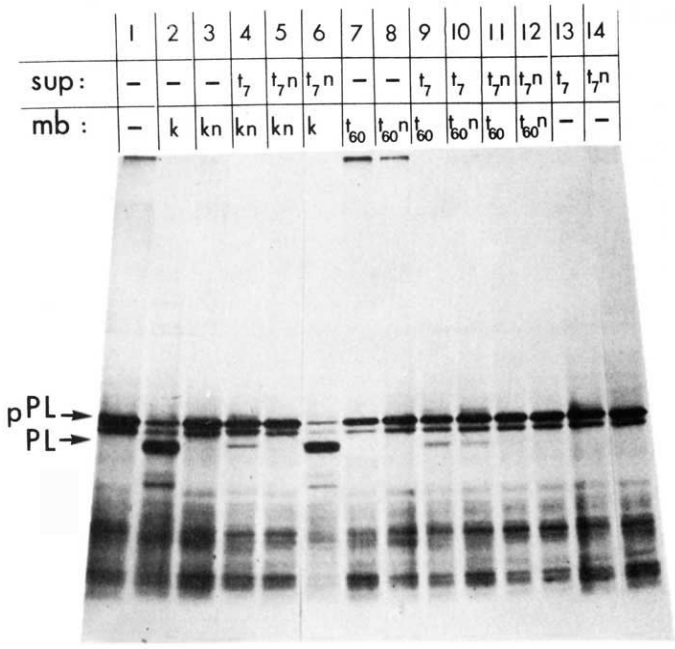


**Fig. 1** The sulphydryl group required for translocation is located on a cytoplasmically disposed, trypsin-sensitive domain of the translocation apparatus. Supernatant (sup) and membrane (mb) fractions were prepared from mildly (7 $\mu g\ ml^{-1}$) and extensively (60 $\mu g\ ml^{-1}$) trypsin-digested samples of EDTA and KCl-stripped rough microsomes (k-mb) as previously described[3]. Aliquots of these fractions were reacted with 1.5 mM *N*-ethylmaleimide for 30 min at 25 °C, then quenched with a 10 fold molar excess of dithiothreitol (DTT). Both modified and unmodified fractions were assayed for their ability to support and/or reconstitute the translocation of preprolactin across the microsomal membrane. Samples of the indicated fractions were added to a wheat-germ *in vitro* translation system[10] programmed with bovine pituitary RNA and supplemented with human placental ribonuclease inhibitor[11] at a final concentration of $0.006 A_{280}$ units $ml^{-1}$. In assaying for the reconstitution of translocation by the t-sup fractions, 3 $\mu l$ of the indicated supernatant fraction were added to a typical 25-$\mu l$ translation mixture. (3 $\mu l$ of t-sup are derived from $0.126 A_{280}$ units of stripped microsomes.) Where indicated, membranes were present at a final concentration of $2.3 A_{280}$ units $ml^{-1}$. Translation products were separated by polyacrylamide gel electrophoresis in $SDS_4$ and detected by autoradiography. Translocation capacity is assessed from the ability of the added fractions to process preprolactin (pPL) to prolactin (PL). The membrane fractions are: k-mb, KCl and EDTA-stripped rough microsomes; kn-mb, *N*-ethylmaleimide-modified k-mb; $t_{60}$-mb, extensively (60 $\mu g\ ml^{-1}$) trypsinized k-mb; $t_{60}$n-mb, *N*-ethylmaleimide-modified $t_{60}$-mb. The supernatant fractions are: $t_7$-sup, supernatant from mildly (7 $\mu g\ ml^{-1}$) trypsinized k-mb; $t_7$n-sup, *N*-ethylmaleimide-modified $t_7$-sup.

* Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755.

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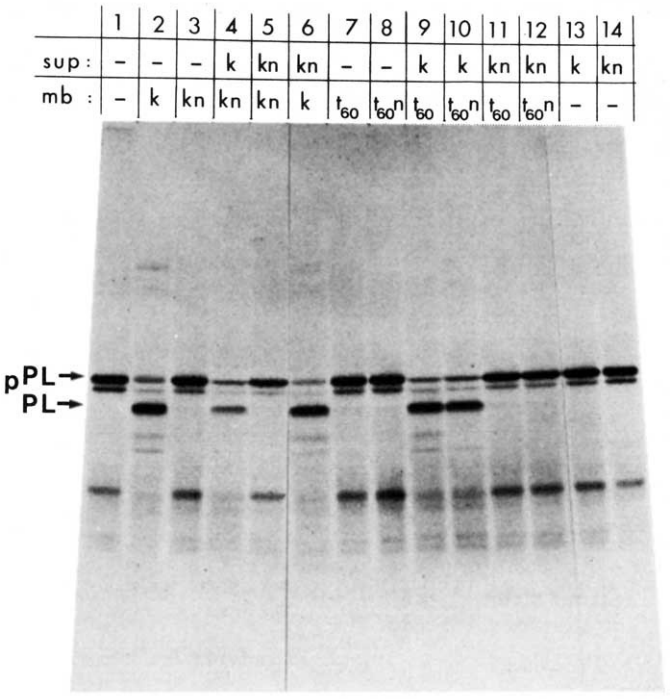


**Fig. 2** A factor removed from EDTA-stripped rough microsomes by extraction with 0.5 M KCl also contains an essential sulphydryl moiety. EDTA-stripped rough microsomes were extracted with 0.5 M KCl, as described by Warren and Dobberstein[2]. Aliquots of the KCl extract (k-sup) and membrane (k-mb) fractions were modified with 1.5 mM *N*-ethylmaleimide as described in Fig. 1 legend. Samples of the modified and unmodified supernatant and membrane fractions were assessed for their ability to support and/or reconstitute translocation activity as indicated by their ability to process preprolactin to prolactin. In assaying for the reconstitution of translocation by the t-sup and k-sup fractions, 3 μl of these supernatant fractions were added to a typical 25-μl translation mixture. (3 μl of k-sup are derived from 0.154 $A_{280}$ units of EDTA-stripped microsomes.) Where indicated, membranes were present at a final concentration of 2.3 $A_{280}$ units $ml^{-1}$. The abbreviations used are: k-sup, 0.5 M KCl extract of EDTA-stripped rough microsomes; kn-sup, *N*-ethylmaleimide-modified k-sup; other abbreviations are as in Fig. 1.

but retains its competence to serve as acceptor for the solubilized fragment(s) and thereby to restore translocation activity (Fig. 1, lane 9). Before assaying for translocation activity, the trypsinized membrane fraction was further incubated in the absence or presence of *N*-ethylmaleimide. The resulting fractions are referred to as $t_{60}$-mb or $t_{60}$n-mb, respectively.

The effects of *N*-ethylmaleimide treatment of these various fractions as well as some essential controls are shown in Fig. 1. Two conclusions can be drawn from the data. First, *N*-ethylmaleimide inactivated only the trypsin-solubilized fragments, but not the membrane-integrated remainder of the translocation apparatus (compare lanes 11, 12 with 9, 10). Thus, the important sulphydryl group(s) is located in the cytosol-exposed, trypsin-sensitive domain of the translocation apparatus. Furthermore, as *N*-ethylmaleimide readily permeates membranes, and as reconstitution of translocation is not affected by *N*-ethylmaleimide modification of $t_{60}$-mb (Fig. 1, lane 10), the residual $t_{60}$-mb cannot contain any sulphydryl groups essential for translocation, that is, the essential sulphydryl is located exclusively on the cytosol-exposed domain of the translocation apparatus. Second, non-trypsinized control membranes (k-mb) must contain membrane-integrated fragments comparable to those that are experimentally generated by trypsinization ($t_{60}$-mb fraction). This was previously suggested by the finding that the translocation activity of trypsin-inactivated membranes could be increased to levels greater than those of untrypsinized membranes by the addition of saturating concentrations of the trypsin-solubilized supernatant factor[3]. Here it is indicated by the finding that a non-trypsinized, but *N*-ethylmaleimide-modified membrane fraction (kn-mb) which is translocation inactive (see Fig. 1, lane 3) can be partially reactivated by a $t_7$-sup fraction (lane 4). In other words, the control,

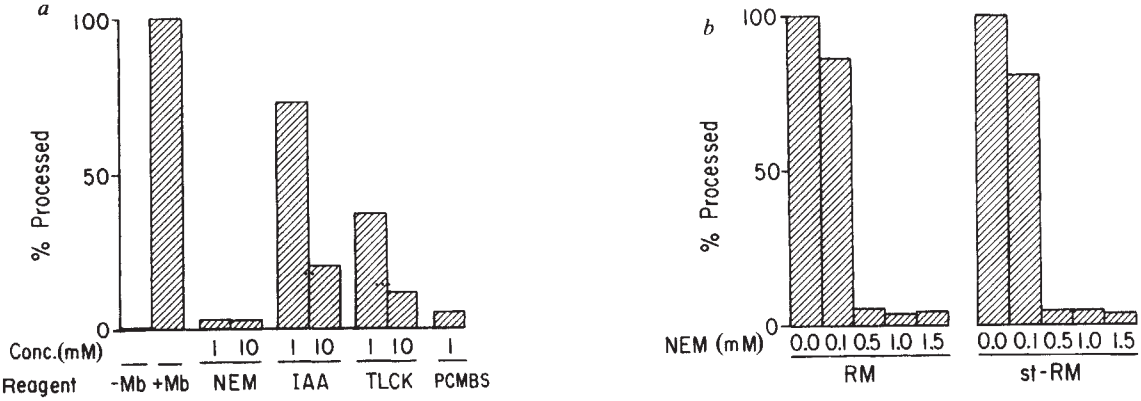


**Fig. 3** *a*, Inhibition of precursor processing (and translocation) by sulphydryl-modifying reagents. Stripped rough microsomes (st-RM) were prepared as previously described[3,12] with the exception that DTT was excluded from all buffers. Modification reactions (50 μl) contained stripped microsomes at a final concentration of 46.5 $A_{280}$ units (determined in 5% SDS), 1% Trasylol, 0.2 M sucrose, 100 mM triethanolamine-HCl (TEA-HCl), *p*H 7.5, 40 mM KCl, 0.8 mM $MgCl_2$ and the indicated concentration of freshly prepared sulphydryl-modifying reagent. Reactions were quenched after 30 min at 25 °C by the addition of at least a 10-fold molar excess of DTT. The DTT-inactivated sulphydryl-modifying reagents were removed from the modified stripped microsomes by centrifugation in a Beckman cellulose nitrate airfuge tube containing a sucrose step gradient comprised of 80 μl of 0.5 M sucrose, 10 mM TEA-HCl, *p*H 7.5, 1 mM DTT and 25 μl of 2.0 M sucrose in the same buffer. After centrifugation (5 min, at 30 lb per sq. inch, 100,000$g_{av}$) the microsomes were removed from the 2.0–0.5 M sucrose interface with a Hamilton syringe, brought to a total volume of 20 μl with 10 mM TEA-HCl, *p*H 7.5, and 1 mM DTT, and assayed for their ability to support translocation (see text) and processing of preprolactin to prolactin. Translocation assays were carried out in a final volume of 50 μl in a wheat-germ translation system[10] supplemented with human placental ribonuclease inhibitor[11] at a final concentration of 0.006 $A_{280}$ units $ml^{-1}$. The wheat-germ system was programmed with pituitary RNA and supplemented with the appropriately modified membranes at a final concentration of 2.3 $A_{280}$ units $ml^{-1}$. The $^{35}$S-Met-labelled products were separated by gel electrophoresis in SDS, detected by autoradiography of the dried gel and quantitated by excising and counting the portion of the gel containing the $^{35}$S-Met-labelled preprolactin and prolactin products[3,12]. Processing is expressed as per cent of control, unmodified membranes (+mb). In this particular experiment, the absolute amount of preprolactin processed by the unmodified membranes was 35.0% of the total preprolactin synthesized. The abbreviations used are: *N*-ethylmaleimide, NEM; iodoacetamide, IAA; *p*-chloromercuribenzene sulphonic acid, PCMBS; *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, TLCK; sodium dodecyl sulphate, SDS. *b*, The processing (and translocation) activity of both rough and stripped rough microsomes is inhibited by *N*-ethylmaleimide modification. A rough microsomal fraction (RM), 107 $A_{280}$ units $ml^{-1}$, was divided into two samples. Ribosomes were removed from the microsomes of one of these samples by mixing the sample with one volume of a ribosome-stripping solution containing 0.25 M sucrose, 40 mM EDTA and 50 mM TEA-HCl, *p*H 7.5. The stripped microsomes (st-RM) were separated from EDTA by centrifugation. Aliquots of both the stripped and the unstripped microsomes were modified with the indicated concentration of *N*-ethylmaleimide (NEM) as described in *a*. After inactivation of residual *N*-ethylmaleimide with DTT, ribosomes were removed from the rough microsomal fractions by EDTA extraction, as described above. The modified, stripped microsomes were separated from inactivated *N*-ethylmaleimide and EDTA by centrifugation and assayed for translocation activity (see *a*). In this particular experiment, the absolute amount of preprolactin processed by the unmodified stripped microsomes was 74.5% of the total preprolactin synthesized.

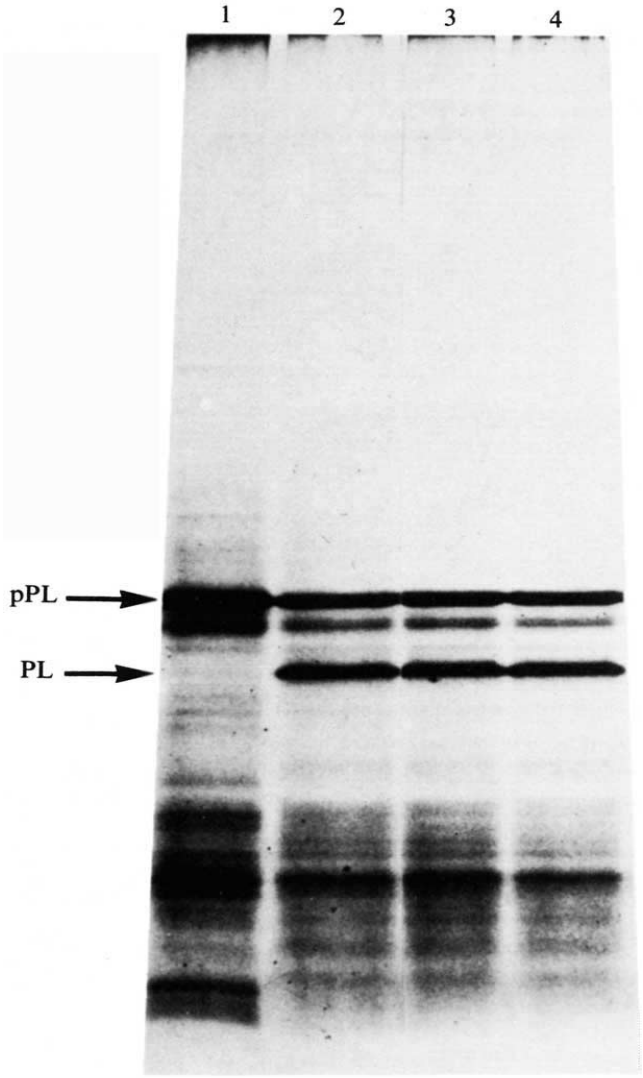


**Fig. 4** Aliquots (40 μl) of stripped microsomes (56 $A_{280}$ units ml$^{-1}$) were modified with the indicated concentration of *N*-ethylmaleimide as described in Fig. 3*a* legend. After termination of the modification reaction with DTT (see Fig. 3*a*), the microsomes were dissociated by the addition of sodium deoxycholate to a final concentration of 0.5% (w/v) and were assayed for the ability of signal peptidase to process full-length $^{35}$S-Met-preprolactin (pPL) as previously described[8]. Lane 1: control, unprocessed preprolactin; lane 2: processing by unmodified, deoxycholate-dissociated microsomes; lanes 3, 4: processing by microsomes which had been modified with 10 mM (lane 3) or 20 mM (lane 4) *N*-ethylmaleimide before deoxycholate dissociation.

untrypsinized membranes behave as if they had already been subjected to limited proteolytic degradation, resulting in the production of some nonfunctional but reactivatable translocation sites. As expected, the observed reactivation of the kn-mb fraction is eliminated when a $t_7$n-sup fraction (Fig. 1, lane 5) is substituted for the $t_7$-sup fraction.

In the controls in Fig. 1, we show that neither the $t_7$-sup (lane 13) nor the $t_7$n-sup (lane 14) fractions are themselves capable of supporting translocation in the absence of added membrane. An additional control (lane 6) demonstrates that the $t_7$n-sup fraction does not inactivate translocation by added k-mb, indicating that our procedures (see Fig. 3*a*) for quenching *N*-ethylmaleimide are effective and that the observed effects of the *N*-ethylmaleimide-modified fractions are not due to a carryover of residual *N*-ethylmaleimide.

The presence of nonfunctional, but reactivatable translocation sites in untrypsinized k-mb suggested that the 'salt factor' of Warren and Dobberstein[2] is in reality a salt-extractable, proteolytic fragment of the translocation apparatus, comparable to that experimentally generated by trypsin. Therefore, one would expect that the salt factor, like the trypsin-generated soluble fragment(s), might also contain a sulphydryl group(s) that is essential for activity. These expectations were indeed borne out by data shown in Fig. 2. Although in our hands KCl extraction does not result in a complete loss of translocation activity, our KCl extract (k-sup) fraction does contain a salt factor which is able to restore translocation activity to trypsin-inactivated microsomes (Fig. 2, lane 9), to *N*-ethylmaleimide inactivated microsomes (Fig. 2, lane 10) and to microsomes inactivated by both trypsin and *N*-ethylmaleimide (Fig. 2, lane 4). In fact, the ability of the KCl extract to restore translocation is quantitatively superior to that of the tryptic extract (compare Figs 1 and 2, lanes 4, 9, 10). Although this difference in activity may reflect a genuine difference in the absolute reactivation capacity of the KCl and tryptic factors, other explanations (particularly tryptic inactivation of a substantial portion of the trypsin-solubilized factor) are also likely. Nevertheless, like its trypsin-generated counterpart, the salt factor is readily inactivated by *N*-ethylmaleimide (Fig. 2, compare lanes 5, 11, 12 with lanes 4, 9, 10). The control (Fig. 2, lane 6) demonstrates that the *N*-ethylmaleimide-inactivated salt factor does not affect translocation of EDTA-stripped, KCl-extracted membranes (Fig. 2, compare lane 6 with lane 3), indicating that the observed effects of the *N*-ethylmaleimide-modified fractions are not due to a carryover of residual *N*-ethylmaleimide.

From these experiments, we conclude that the translocation-reactivating factors present in k-sup and $t_7$-sup are functionally and structurally similar, in that both restore translocation activity to *N*-ethylmaleimide- or trypsin-inactivated test membranes, and both contain an essential sulphydryl group(s). Although both these factors are probably derived from the same parent molecule, differences in their extractability (the trypsin factor is released in the absence of salt) suggest that tryptic digestion removes a smaller fragment of the parent translocator protein than is generated by the endogenous tissue protease(s) which produces the salt factor. Presumably, it is this additional domain of the salt factor which is responsible for the differential salt extractability of the salt and tryptic factors. In any case, the observation that the cytoplasmically disposed domain of the translocation apparatus contains an essential sulphydryl which can be readily modified provides us with a tool which should be extremely useful in the purification and characterization of the polypeptides responsible for translocation of nascent secretory and membrane proteins across the membrane of the rough endoplasmic reticulum.

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# Efficiency of the adaptive response of *Escherichia coli* to alkylating agents

**John Cairns**

Imperial Cancer Research Fund, Burtonhole Lane, London NW7 1AD, UK

**When cultures of *Escherichia coli* are exposed to a low level of the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) they accumulate mutations for about 20 min and then**

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